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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Julie A. Kiriara et al.
Title: METHOD FOR ALTERING THE NUTRITIONAL CONTENT OF PLANT SEED
Attorney Docket No.: 950.011US2

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PATENT APPLICATION TRANSMITTAL

BOX PATENT APPLICATION

Commissioner for Patents
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We are transmitting herewith the following attached items and information (as indicated with an "X"):

- ☒ Return postcard.
☒ **DIVISIONAL** of prior Patent Application No. 08/763,704 (under 37 CFR § 1.53(b)) comprising:
☒ Specification (122 pgs, including claims numbered 1 through 100 and a 1 page Abstract).
☒ Informal Drawing(s) (11 sheets).
☒ Copy of signed Combined Declaration and Power of Attorney (3 pgs) from prior application.
☒ Incorporation by Reference: *The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied herewith, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.*
☒ Authorization to charge the amount of \$1,058.00 to pay the filing fee to Deposit Account No. 19-0743.
☒ Prior application is assigned of record to DEKALB Genetics Corporation.
☒ Information Disclosure Statement (1 pgs), Form 1449 (60 pgs). References NOT enclosed, cited in prior application.
☒ Preliminary Amendment (3 pgs).

The filing fee has been calculated below as follows:

	No. Filed	No. Extra	Rate	Fee
TOTAL CLAIMS	26 - 20 =	6	x 18 =	\$108.00
INDEPENDENT CLAIMS	2 - 3 =	0	x 78 =	\$0.00
[X] MULTIPLE DEPENDENT CLAIMS PRESENTED				\$260.00
BASIC FEE				\$690.00
TOTAL				\$1,058.00

Please charge Deposit Account No. 19-0743 in the amount of \$1,058.00 to pay the filing fee for this application. Please charge any additional required fees or credit overpayment to Deposit Account No. 19-0743.

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By: Dawn M. Poole

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S/N 08/763,704

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Julie A. Kiriara et al.	Examiner:	Gary Benzion
Serial No.:	Unknown	Group Art Unit:	1649
Filed:	Herewith	Docket:	950.011US2
Title:	METHOD FOR ALTERING THE NUTRITIONAL CONTENT OF PLANT SEED		

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

When the above-identified patent application is taken up for consideration, please amend the application as follows:

IN THE SPECIFICATION

On page 1, line 5, after "This application is a" please insert -- divisional of U.S. Serial No. 08/763,704, filed December 9, 1996 which is a --.

IN THE CLAIMS

Please cancel claims 1-71; 74-77; 80-83; 85; 87; and 92-93.

Please amend the claims as follows:

78. (Amended) A seed derived from the plant of claim 72 or 73.
84. (Amended) The transgenic plant of claim 72 or 73, wherein the promoter comprises the 10 kD zein promoter.
86. (Amended) The transgenic plant of claim 72 or 73, wherein the promoter comprises the 27 kD zein promoter.
88. (Amended) The transgenic plant of claim 72 or 73, wherein the preselected DNA sequence, which encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding a seed storage protein, encodes an RNA molecule substantially complementary

to all or a portion of a mRNA encoding 19 kD α -zein protein.

89. (Amended) The transgenic plant of claim 72 or 73, wherein the preselected DNA sequence, which encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding a seed storage protein, encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding a 22 kD α -zein protein.

90. (Amended) The transgenic plant of claim 72 or 73, wherein the preselected DNA sequence, which encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a seed storage protein, encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a 19 kD α -zein protein.

91. (Amended) The transgenic plant of claim 72 or 73, wherein the preselected DNA sequence, which encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a seed storage protein, encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a 22 kD α -zein protein.

94. (Amended) The transgenic plant of claim 72 or 73, further comprising stably transforming the cells with a gene which encodes kernel hardness.

95. (Amended) The transgenic plant of claim 72 or 73, wherein the cell is transformed by a method selected from the group consisting of electroporation, microinjection, microprojectile bombardment, and liposomal encapsulation.

100. (Amended) The transgenic plant of claim 78 or 79, further comprising stably transforming the cells with at least one selectable marker gene.

PRELIMINARY AMENDMENT

Serial Number: Unknown

Filing Date: Filed Herewith

Title: METHOD FOR ALTERING THE NUTRITIONAL CONTENT OF PLANT SEED

Page 3

Dkt: 950.011US

REMARKS

Claims 1-71, 74-77, 80-83, 85, 87, and 92-93 have been canceled, claims 72, 73, 78-79, 84, 86, 88-91, 94-95 and 100 are now pending. The amendments to the claims are made to reflect the proper dependencies due to the cancellation of certain claims, and are not intended to surrender any range of equivalents to which the amended claims may be entitled, such as equivalents of any claim element which are not within the prior art. Please enter into the record the preliminary amendment described herein prior to examination and consideration of the above-identified application.

When the Examiner takes the above-identified application up for the first Office Action, consideration of these amendments and remarks is respectfully requested. The Examiner is invited to telephone the below-signed attorney at 612-373-6903 to discuss any questions which may remain with respect to the present Application.

Respectfully submitted,

JULIE A. KIRIHARA ET AL.

By their Representatives,

SCHWEGMAN, LUNDBERG, WOESSNER & KLUTH, P.A.

P.O. Box 2938

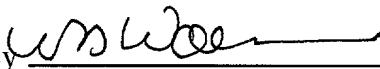
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23 June 00

By



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"Express Mail" mailing label number: EL576922808US

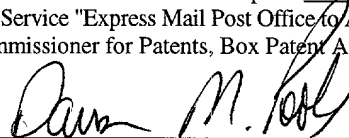
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By:

Dawn M. Poole

Signature:



METHOD FOR ALTERING THE NUTRITIONAL CONTENT OF PLANT SEED

Cross-Reference to Related Applications

5 This application is a continuation-in-part of U.S. application Serial No. 08/112,245, filed August 25, 1993, currently pending, the disclosure of which is incorporated by reference herein.

Field of the Invention

10 The invention relates generally to modification of the nutritional content of maize seed utilizing preselected DNA constructs. More specifically, the invention relates to the use of preselected DNA constructs to transform maize plants so as to alter the levels of proteins, such as seed storage proteins, e.g., the prolamines (zeins), in the seeds of transgenic maize plants. Thus, the invention provides a
15 mechanism to replace nutritionally deficient proteins with nutritionally enhanced proteins, and/or to increase the levels of starch, in the seed of transgenic maize plants.

Background of the Invention

20 In agriculturally important seed crops, the expression of storage protein genes directly affects the nutritional quality of the seed protein. In maize, the prolamine (zein) fraction of storage proteins comprises over 50% of the total protein in the mature seed. The zeins designated α -zein are especially abundant. The α -zein polypeptides contain extremely low levels of the essential amino acids lysine and
25 tryptophan. Thus, maize seed protein is deficient in these amino acids because such a large proportion of the total seed storage protein is contributed by the α -zeins (Mertz et al., 1964).

 The development of breeding steps to improve maize based on the manipulation of zein profile is hampered by the complexity of the zein proteins. The
30 term "zein" encompasses a family of some 100 related proteins. Zeins can be

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divided into four structurally distinct types: α -zeins include proteins with molecular weights of 19,000 and 22,000 daltons; β -zeins include proteins with a molecular weight of 14,000 daltons; γ -zeins include proteins with molecular weights of 27,000 and 26,000 daltons; and δ -zeins include proteins having a molecular weight of 10,000 daltons. The α -zeins are the major zein proteins found in the endosperm of maize kernels. However, the complexity of zein proteins goes beyond these size classes. Protein sequence analyses indicates that there is microheterogeneity in zein amino acid sequences. This is in accord with isoelectric focusing analyses which show charge differences in zein proteins. Over 70 genes encoding the zein proteins have been identified (Rubenstein, 1982), and the zein genes appear to be located on at least three chromosomes. Thus, the zein proteins are encoded by a multigene family.

Based on sequence and hybridization data, the zein multigene family is divided into several subfamilies. Each subfamily is defined by sequence homology to a cDNA clone: A20, A30, B49, B59, or B36. Hybrid-select translation studies which employ B49 and B36 select mRNAs that code for predominantly heavy class (23 kD) α -zein proteins, while A20, A30, and B59 select for predominantly the light class (19 kD) α -zein proteins (Heidecker and Messing, 1986). A comparison of zein sequences in each of the subfamilies A20, A30 and B49 have identified four distinct functional domains (Messing et al., 1983). Region I corresponds to the signal peptide present in most, if not all, zeins. Regions II and IV correspond to the amino and carboxyl termini, respectively, of the mature zein protein. Region III corresponds to the coding region between Regions II and IV, including a region which has tandem repeats of a 20 amino acid sequence.

25 There are several mutations known to cause reductions in zein synthesis that lead to alterations in the amino acid content of the seed. For example, in the seeds of plants homozygous for the recessive mutation *opaque-2*, the zein content is reduced by approximately 50% (Tsai et al., 1978). The *opaque-2* mutation primarily affects synthesis of the 19 and 22 kD α -zein proteins, causing a significant

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1988c; Rodermel et al., 1988). However, to date there has been no description of attempts to use antisense technology to alter the nutritional content of seeds.

Down regulation of gene expression in a plant may also occur through expression of a particular transgene. This type of down regulation is referred to as co-suppression and involves coordinate silencing of a transgene and a second transgene or a homologous endogenous gene (Matzke and Matzke, 1995). For example, cosuppression of a herbicide resistance gene in tobacco (Brandle et al., 1995), polygalacturonidase in tomato (Flavell, 1994) and chalcone synthase in petunia (U. S. Patent No. 5,034,323) have been demonstrated. Flavell (1994) suggested that multicopy genes, or gene families, must have evolved to avoid cosuppression in order for multiple copies of related genes to be expressed in a plant.

Thus, there is a need for a method to alter the nutritional content of seeds and produce kernels with good agronomic characteristics, including maintaining kernel hardness, yield, and disease resistance of the parent genotype. Furthermore, there is a need for a method to decrease expression of seed storage proteins of poor nutritional quality while increasing proteins with higher contents of nutritionally advantageous amino acids, such as methionine and lysine, and/or while increasing the starch content of seeds.

Summary of the Invention

The invention provides methods which employ a genetically engineered, preselected DNA sequences or segments to alter the nutritional content of plant seeds. The expression of said preselected DNA sequence results in an altered protein and/or amino acid composition in the transgenic plant, plant tissue, plant part, or plant cell relative to the corresponding nontransformed, i.e., nontransgenic, plant, plant tissue, plant part, or plant cell. Preferably, the seeds of said transgenic plant have an increased amount, e.g., weight percent, of at least one amino acid essential to the diet of animals, relative to nontransformed, i.e., nontransgenic, seeds. An increase in the weight percent of at least one amino acid

essential to the diet of animals, e.g., lysine, methionine, isoleucine, tryptophan, or threonine, in seeds increases the nutritional value of those seeds for animal, e.g., feeds for poultry and swine, or human consumption.

Thus, the invention provides a method which comprises stably transforming cells of a plant with an expression cassette. The expression cassette comprises a preselected DNA sequence which codes for an RNA molecule which is substantially identical (sense), or complementary (antisense), to all or a portion of a messenger RNA ("target" mRNA), i.e., an endogenous or "native" mRNA, which is present in a nontransformed plant cell. The target mRNA encodes a plant seed storage protein, preferably a protein which is deficient in at least one amino acid, and more preferably deficient in an amino acid which is essential to the diet of animals.

The resultant transformed cells are used to regenerate fertile transgenic plants which in turn yield transgenic seeds, wherein the preselected DNA sequence is expressed in the transgenic seeds in an amount effective to substantially reduce or decrease the amount, weight percent or level of a seed storage protein relative to the amount, weight percent or level of said seed storage protein present in the corresponding nontransgenic seeds, e.g., seeds of a nontransformed RO control plant or corresponding nontransformed seeds isolated from the transgenic plant. The seed storage protein is one which is deficient in at least one amino acid essential to the diet of an animal. Preferably, the decrease in the amount of the seed storage protein results in an increase in the weight percent of seed storage proteins comprising higher percentages of nutritionally advantageous amino acids. The preselected DNA sequence preferably codes for an RNA molecule substantially complementary to all or a portion of a mRNA coding for a 19 kD or 22 kD α -zein protein. A reduction in seed storage proteins, e.g., the α -zeins, may be accompanied by a decrease in the degree of kernel hardness. Hardness of the kernel may be enhanced in these cases by modification of the kernel phenotype as described for the *opaque-2* mutation (Lopes and Larkins, 1991) or by genetically modifying plants to increase the levels of certain endosperm proteins such as the 27 kD γ -zein.

The genetically engineered DNA sequences of the invention are “preselected” in that the coding regions contained therein have been isolated *in vitro*, and identified at least functionally. Thus, a “preselected” DNA is a DNA sequence or segment that has been isolated from a cell, purified, and amplified. The choice of the preselected DNA sequence will be based on the amino acid composition of the polypeptide encoded by the sense strand of a preselected DNA sequence, and preferably, the ability of the polypeptide to accumulate in seeds. Preferably, the number of said coding regions has also been ascertained. Also preferably, the isolated DNA molecule is “recombinant” in that it contains preselected DNA sequences from different sources which, preferably, have been linked to yield chimeric expression cassettes. The preselected DNA sequences are preferably about 2-3 kb.

The invention further provides a method to increase the starch content of a plant, plant part, plant tissue or plant cell. The method comprises stably transforming cells of a plant with an expression cassette. The expression cassette comprises a preselected DNA sequence coding for an RNA molecule substantially identical, or complementary, to all or a portion of at least one mRNA coding for a plant seed storage protein. Preferably, the preselected DNA sequence is operably linked to a promoter functional in a plant and/or seed. Transformed cells are used to regenerate fertile transgenic plants and seeds. The preselected DNA sequence is preferably expressed in the transgenic seeds in an amount effective to decrease the weight percent of seed storage protein in the transgenic seed over the weight percent of seed storage protein present in the corresponding nontransgenic seed. The preselected DNA sequence is also preferably expressed in the transgenic seeds in an amount effective to increase the weight percent of starch in the transgenic seed over the weight percent of starch present in the corresponding nontransgenic seed. An increase in the weight percent of the starch of seeds improves the food value of the seed, or its value as a source of starch for use in processed food products or in

compared to other seed storage proteins. The second preselected DNA sequence encodes a polypeptide of desired amino acid composition, i.e., a polypeptide comprising at least one amino acid essential to the diet of animals. The polypeptide, preferably, has physical properties which minimize disruption of seed cellular structure and therefore grain quality. It is preferred that each preselected DNA sequence is operably linked to a promoter functional in a plant and/or seed.

Following transformation, transformed plant cells having the first and second preselected DNA sequences stably, i.e., chromosomally, integrated into their genome are selected and used to regenerate fertile transgenic plants and seeds. The transgenic seeds are characterized by the expression of the first DNA sequence in an amount effective to substantially reduce or decrease the amount, weight percent, or level, of the undesirable seed storage protein, or an amino acid present in said protein, over the amount, weight percent, or level, of that seed storage protein, or the amino acid present in that protein, which is present in nontransgenic seeds. The transgenic seeds are also preferably characterized by the expression of the second DNA sequence as a plant protein in an amount effective to yield an increase in the amount, weight percent or level of at least one amino acid essential to the diet of animals over the amount, weight percent or level of that amino acid present in nontransgenic seeds.

In a preferred embodiment, the expression of the first preselected DNA sequence in transgenic maize seed inhibits the weight percent of 19 kD or 22 kD α -zein. In another preferred embodiment, the expression of the second preselected DNA sequence in transgenic seed results in an increase in the weight percent of a 10 kD δ -zein protein. In yet another preferred embodiment, the expression of the second preselected DNA sequence in transgenic seed results in an increase in the weight percent of a 27 kD zein protein. In yet another preferred embodiment, the second preselected DNA encodes a synthetic polypeptide, such as MB1 (Beauregard et al., 1995). MB1 is a stable synthetic polypeptide highly enriched in amino acids essential for animal nutrition (e.g., methionine, threonine,

lysine, and leucine) which also adopts an α -helical conformation. The synthetic polypeptide MB1 shares some properties of maize zein proteins, e.g., MB1 is alcohol soluble and contains multiple α -helical domains. However, other polypeptides, synthetic and naturally occurring, with preselected desired amino acid compositions, and genes coding therefor, could be employed in the practice of the invention. As used herein, the term "polypeptide" includes protein.

The invention also provides a method to increase the amount, weight percent or level of a polypeptide in a plant. The method comprises stably transforming plants, plant cells, plant tissue or plant parts with a first preselected DNA sequence which encodes a seed storage protein and a second preselected DNA sequence which encodes at least a portion of a preselected, desired polypeptide. The polypeptide may be encoded by the genome of the nontransformed plant or plant cell ("endogenous" or "native"), or, alternatively may not be native to, i.e., present in, the genome of the nontransformed "wild type" plant or plant cell (termed "heterologous," "non-native" or "foreign"). Preferably, the second preselected DNA sequence encodes a bacterial enzyme, e.g., AK, DHDPS, EPSPS, a bacterial toxin, e.g., the crystal toxin from Bt, a seed storage protein, e.g., Z27, or a non-maize seed storage protein, such as nut and legume seed storage proteins. See, for example, U.S. Patent No. 4,769,061; U.S. Patent No. 4,971,908; PCT/US90/04462; PCT/WO89/11789; and Altenbach et al. (1989).

Transformed plant cells having the first and second preselected DNA sequences stably, i.e., chromosomally, integrated therein are selected and used to regenerate fertile transgenic plants and seeds. Transgenic seeds of the invention are characterized by substantial inhibition of the expression of at least one seed storage protein. The second preselected DNA sequence is expressed in said transgenic seeds in an amount effective to increase the weight percent of at least one amino acid present in polypeptide encoded by the second preselected DNA sequence relative to the weight percent of that amino acid in nontransgenic seeds. Alternatively, the second preselected DNA sequence is expressed in transgenic seed in an amount

transformant (GW01) carrying pDPG340 and pDPG380 to nontransformed inbreds, and R2 self-pollinations. Lanes 1-8 contain zein extracts from R2 kernels crossed to CN in the R1 generation and self-pollinated in the second generation. Lane 9 contains zein extract from untransformed CN. Lanes 10-17 contain zein extracts from R2 kernels crossed to AW in the first generation and self-pollinated in the second generation. Lane 18 contains zein extract from untransformed AW. Lane 19 contains molecular weight markers.

Figure 6 shows SDS-PAGE analysis of zein extracts of vitreous or opaque kernels from segregating populations resulting from crosses of hemizygous pDPG530 transformants to untransformed inbreds AW and CV. KP014 x AW (lanes 1-2); AW x KP014 (lanes 3-4); KP015 x AW (lanes 5-6); AW x KP015 (lanes 7-8); CV x KP015 (lanes 9-10); AW x KP015 (lanes 11-12). Lanes 13-19 are AW, CV, ILP, IHP, AK835 opaque, AK835 normal, and W64A opaque, respectively. Lane 20 contains molecular weight markers.

Figure 7 shows SDS-PAGE analysis of zein extracts of proteins from individual kernels of segregating populations resulting from crosses of hemizygous transformants and untransformed inbreds. pDPG530 transformant KP015 (AW x KP015, lanes 1-2; CV x KP015, lanes 3-4; KP015 x AW, lanes 5-6, and KP016 (CV x KP016, lanes 7-8; KP016 x AW, lanes 9-10) and pDPG531 transformant KQ018 (KQ018 x AW, lanes 11-12). Lanes 13-18 are untransformed controls CW, AR, CV, AW, W64A, O2 and W64A, respectively. Lanes 19-20 contain molecular weight markers.

Figure 8 shows α -zein mRNA levels in developing kernels from a segregating population resulting from crosses of hemizygous pDPG530 and pDPG531 transformants to untransformed inbreds AW and CV. AW x KP015 (pDPG530 transformant; lanes 1-10; top panel); KP015 x AW (pDPG530 transformant; lanes 11-20; top panel); CV x KP015 (pDPG530 transformant; lanes 1-10; lower panel); and KQ012 x AW (pDPG531 transformant; lanes 11-20; lower panel). Kernels were isolated 21 days post-pollination.

Figure 10 shows SDS-PAGE analysis of zein extracts from segregating populations resulting from crosses of pDPG531 transformants to untransformed inbreds AW and CV. CV x KQ012 (lanes 1-4); KQ012 x AW (lanes 5-8); KQ020 x AW (lanes 13-15); KQ020 x CV (lanes 16-19). Controls CW, AR, CV and AW (lanes 9-12, respectively). Lane 20 contains a molecular weight marker.

10 Definitions

As used herein, “substantially complementary” means that two nucleic acid sequences have at least about 65%, preferably about 70%, more preferably about 90%, and even more preferably about 98%, sequence complementarity to each other. A substantially complementary RNA molecule is one that has sufficient sequence complementarity to the mRNA encoding a seed storage protein to result in a reduction or inhibition of the translation of the mRNA.

25 As used herein, “substantial reduction,” or “substantial decrease” means that a transgenic plant, plant part, plant cell or plant tissue has a reduced or decreased amount, level or weight percent of a particular amino acid, or polypeptide, relative to the amount, level or weight percent of that amino acid, or polypeptide, in the corresponding nontransgenic plant, plant part, plant cell or plant tissue.

5 polypeptide, in the corresponding nontransgenic plant, plant part, plant cell or plant tissue.

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As used herein, "genetically modified" or "transgenic" means a plant cell, plant part, plant tissue or plant which comprises a preselected DNA segment

which is introduced into the genome of a plant cell, plant part, plant tissue or plant by transformation. The term "wild type" refers to an untransformed plant cell, plant part, plant tissue or plant, i.e., one where the genome has not been altered by the presence of the preselected DNA segment.

5 As used herein, "plant" refers to either a whole plant, a plant tissue, a plant part, such as pollen or an embryo, a plant cell, or a group of plant cells. The class of plants which can be used in the method of the invention is generally as broad as the class of seed-bearing higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants. Seeds derived from
10 plants regenerated from transformed plant cells, plant parts or plant tissues, or progeny derived from the regenerated transformed plants, may be used directly as feed or food, or can be altered by further processing. In the practice of the present invention, the most preferred plant seed is that of corn or *Zea mays*. The transformation of the plants in accordance with the invention may be carried out in
15 essentially any of the various ways known to those skilled in the art of plant molecular biology. These include, but are not limited to, microprojectile bombardment, microinjection, electroporation of protoplasts or cells comprising partial cell walls, and *Agrobacterium*-mediated DNA transfer.

 As used herein, the term "a seed storage protein deficient in at least
20 one amino acid that is essential to the diet of an animal" means that the protein has a lower than average weight percent of at least one amino acid which is essential to the diet of an animal. Amino acids which are essential to the diet of animals include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Preferred amino acids which are essential in the diet of
25 animals include methionine, threonine, lysine, isoleucine, tryptophan, and mixtures thereof. A plant seed storage protein can contain one or more of these essential amino acids. For example, the average weight percent of lysine in a maize seed is about about 0.24-0.26%. Thus, a seed storage protein, such as an α -zein, which

does not comprise lysine, is deficient in lysine. The average weight percent of a particular amino acid is determined by methods well known to the art.

As used herein, "isolated" means either physically isolated from the cell or synthesized *in vitro* in the basis of the sequence of an isolated DNA segment.

As used herein, a "native" gene means a DNA sequence or segment that has not been manipulated *in vitro*, i.e., has not been isolated, purified, and amplified.

I. DNA Molecules of the Invention

A. Isolation of Preselected Sense and Antisense DNA Sequences

1. α -Zein Seed Storage Proteins

A genetically engineered, isolated purified DNA molecule useful in the invention can comprise a preselected DNA sequence encoding an RNA molecule substantially homologous, or complementary, to all or a portion thereof of a mRNA coding for a plant seed storage protein, e.g., one of the α -zein proteins. As used herein, a "seed storage protein" is a protein which is one of the major proteins in mature seeds of plants such as maize, and comprises a signal peptide sequence at the amino terminal end of the pre-form of the protein, and which comprises a tandem repeat of amino acid sequences in the mature form of the protein.

Plant seed storage proteins or zein proteins include, but are not limited to, zein proteins, such as α -zeins, e.g., proteins of 19,000 and 22,000 daltons; β -zein proteins, e.g., proteins with a molecular weight of 14,000 daltons; γ -zein proteins, e.g., proteins with molecular weights of 27,000 and 16,000 daltons; and δ -zein proteins, i.e., proteins with a molecular weight of 10,000 daltons. Certain seed storage proteins are deficient in at least one amino acid essential to the diet of animals. For example, the 19 kD and 22 kD α -zein proteins contain low levels of the amino acids lysine and tryptophan which are essential to the diet of animals.

The preselected sense or antisense DNA sequence can encode an RNA molecule preferably having about 15 nucleotides to 2,000 nucleotides and more preferably about 50-1,000 nucleotides. The DNA sequence can be derived from the 5' terminus or the 3' terminus and can include all or only a portion of the coding and/or noncoding regions. It will be understood by those of skill in the art that a sense or antisense DNA sequence should provide an RNA sequence having at least about 15 nucleotides in order to provide for substantial inhibition of the expression of the mRNA coding for the seed storage protein.

The preselected DNA sequences of the invention are obtained by cloning a DNA molecule, sequence or segment which encodes, and can be expressed as a mRNA of, a seed storage protein. Portions of the preselected DNA sequence
25 can also include noncoding nucleotides located at either the 5' or 3' ends of the sense coding sequence. A preselected DNA sequence which encodes an RNA sequence that is substantially complementary to a mRNA sequence encoding a seed storage protein is typically a "sense" DNA sequence cloned in the opposite orientation (i.e., 3' to 5' rather than 5' to 3'). A sense DNA sequence encoding a seed storage protein

can be cloned using standard methods as described in Sambrook et al. (1989), and U.S. Patent No. 5,508,468.

A subfragment of a preselected DNA sequence which encodes a full-length seed storage protein can be generated using restriction enzymes. The subfragment is preferably selected based upon the known functional domains of seed storage proteins. A seed storage protein has at least four different functional domains: a signal peptide domain, a domain which includes the amino terminal portion of the mature protein which is located downstream of the signal peptide, a domain which includes tandem repeats of a 20 amino acid sequence which is located downstream of the amino terminus of the mature protein, and a domain which includes the carboxy terminus of the protein. The size and location of these functional domains in the α -zein proteins are shown in Figure 1 and can be determined for other seed storage proteins by comparing the amino acid sequence of other seed storage proteins to the amino acid sequence of the α -zein proteins.

Suitable examples of preselected DNA sequences that can provide all or a portion of a sense or antisense seed storage protein, e.g., α -zein, DNA sequence include cDNA clones A20, A30, B49, B59, B36, Z4, and Z15 prepared as described by Messing et al. (1983). Preferred cDNA clones are an A20 clone, which encodes a 19 kD α -zein protein, and a Z4 clone, which encodes a 22 kD α -zein protein. Portions of the Z4 and the A20 DNA sequences can be generated with restriction endonucleases.

It is also contemplated that preselected DNA sequences homologous or complementary to any portion of the A20 or Z4 RNA, in vectors appropriate for expression in plants, may be used to substantially decrease the production of seed storage proteins. Examples of such DNA sequences are sequences which may be homologous or complementary to the 5' region of the DNA or RNA sequence such as the 3' region of the promoter and the cap site (Figure 4A), or the 3' region of the gene such as the AATAAA-like polyadenylation signal, upstream of the poly(A) tail (Figure 4C). It is further contemplated that a preselected DNA sequence

homologous or complementary to a conserved domain common to more than one gene in a gene family or subfamily, such as domain IIIB or one or more of the other domains shown in Figure 1, may also be useful to substantially inhibit the expression of members of the gene family or subfamily (Figure 4B). It is further contemplated that the preselected DNA sequence may encode an RNA molecule which is substantially identical to all or a portion of a mRNA encoding a seed storage protein, e.g., a preselected DNA sequence encoding a RNA molecule substantially identical to the mRNA encoding 10 kD zein, 27 kD zein, or MB1.

In a preferred embodiment, a sense DNA sequence encoding a 19 kD α -zein protein and/or a sense DNA sequence encoding a 22 kD α -zein protein is prepared from a cDNA library generated from endosperm tissue as described in Hu et al. (1982) and Geraghty et al. (1982), which are hereby incorporated by reference. The cDNA clones encoding a 19 kD α -zein protein and/or a 22 kD α -zein protein can be characterized by standard methods such as DNA hybridization or detection of gene expression by immunotechniques including Western blot analysis. The presence of the coding sequence of the 19 kD or 22 kD α -zein protein can be confirmed by DNA sequencing.

2. Other Preselected DNA Sequences

Another preselected DNA sequence useful in the method of the invention encodes a polypeptide, including a plant protein, comprising at least one amino acid essential to the diet of animals operably linked to a promoter functional in a plant and/or seeds. The expression of the preselected DNA sequence, coding for the polypeptide comprising at least one amino acid essential to the diet of animals, in a plant cell provides for an increase in expression of the polypeptide so that the weight percent of the amino acid residue is substantially increased in the plant regenerated from the transformed plant cell, or seed derived from said plant, over the amount normally present in the corresponding untransformed plant or seed. Preferably, the preselected DNA sequence is co-transformed into plant cells with a

second preselected antisense or sense DNA sequence, the expression of which results in the inhibition of expression of a seed storage protein relatively deficient in an amino acid essential in the diet of animals.

The preselected DNA sequence coding for a polypeptide comprising
 5 at least one amino acid essential in the diet of animals may be a polypeptide expressed in a plant seed, such as a 10 kD zein protein. Other polypeptides that contain one or more amino acid residues essential in the diet of animals include the synthetic polypeptide MB1 (Beauregard et al., 1995). It is contemplated that any
 10 gene encoding a naturally occurring polypeptide, or a synthetic polypeptide, that contains at least one amino acid essential in the diet of an animal may be used in the present invention. The Z10 and MB1 proteins are illustrative of a naturally occurring protein and a synthetic polypeptide, respectively, although one of skill in the art will realize that many other proteins are useful in the practice of the present invention.

The preselected DNA sequences encoding these polypeptides can be
 15 obtained by standard methods, as described by Sambrook et al., cited *supra*. For example, a cDNA clone encoding a 10 kD zein protein can be obtained from maize endosperm tissue, as described by Kirihara et al. (1988). The DNA sequence is then preferably combined with a promoter that is functional in plant cells or seeds. The preferred promoter is a promoter functional during plant seed development, such as
 20 the Z27 or Z10 promoter.

The gene encoding the synthetic polypeptide MB1 is obtained from Mary A. Hefford (Center for Food and Animal Research, Agriculture and Agri-Food Canada). The preselected DNA sequence encoding a synthetic polypeptide such as MB1 is operably linked to a signal sequence derived from a seed storage protein.
 25 For example, the MB1 DNA sequence can be operably linked to the 15 kD zein signal peptide sequence.

It is also contemplated that a preselected DNA sequence encodes a desirable seed storage protein. Thus, the expression of a first preselected DNA sequence can inhibit the expression of an undesirable seed storage protein, while the

expression of a second preselected DNA sequence can encode a desirable gene product, e.g., a desirable seed storage protein. For example, it is envisioned that the expression of the first preselected DNA sequence, which comprises partial gene DNA sequences, may be advantageous for the suppression of the expression of undesirable seed storage proteins, if those partial DNA sequences target DNA or RNA sequences not present in the second preselected DNA sequence which encodes a desirable polypeptide, e.g., 10 kD zein or MB1, in order to avoid suppression of expression of the desirable polypeptide.

B. Optional Sequences for Expression Cassettes

1. Promoters

Preferably, the preselected DNA sequence of the invention is operably linked to a promoter, which provides for expression of the preselected DNA sequence. The promoter is preferably a promoter functional in plants and/or seeds, and more preferably a promoter functional during plant seed development. A preselected DNA sequence is operably linked to the promoter when it is located downstream from the promoter, to form an expression cassette.

Most endogenous genes have regions of DNA that are known as promoters, which regulate gene expression. Promoter regions are typically found in the flanking DNA upstream from the coding sequence in both prokaryotic and eukaryotic cells. A promoter sequence provides for regulation of transcription of the downstream gene sequence and typically includes from about 50 to about 2,000 nucleotide base pairs. Promoter sequences also contain regulatory sequences such as enhancer sequences that can influence the level of gene expression. Some isolated promoter sequences can provide for gene expression of heterologous DNAs, that is a DNA different from the native or homologous DNA.

Promoter sequences are also known to be strong or weak, or inducible. A strong promoter provides for a high level of gene expression, whereas a weak promoter provides for a very low level of gene expression. An inducible promoter is a promoter that provides for the turning on and off of gene expression in

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promoter and control elements of corresponding genomic clones can then be localized using techniques well known to those of skill in the art.

A preselected DNA sequence can be combined with the promoter by standard methods as described in Sambrook et al., cited *supra*, to yield an expression cassette. Briefly, a plasmid containing a promoter such as the 35S CaMV promoter can be constructed as described in Jefferson (1987) or obtained from Clontech Lab in Palo Alto, California (e.g., pBI121 or pBI221). Typically, these plasmids are constructed to have multiple cloning sites having specificity for different restriction enzymes downstream from the promoter. The preselected DNA sequence can be subcloned downstream from the promoter using restriction enzymes and positioned to ensure that the DNA is inserted in proper orientation with respect to the promoter so that the DNA can be expressed as sense or antisense RNA. Once the preselected DNA sequence is operably linked to a promoter, the expression cassette so formed can be subcloned into a plasmid or other vector.

Once the preselected sense DNA sequence is obtained, all or a portion of the DNA sequence can be subcloned into an expression vector (see below) in the opposite orientation (i.e., 3' to 5'). Similarly, all or a portion of the preselected DNA sequence can be subcloned in sense orientation (i.e., 5' to 3'). The preselected DNA sequence is subcloned downstream from a promoter to form an expression cassette.

In a preferred embodiment, a cDNA clone encoding a Z4 22 kD α -zein protein is isolated from maize endosperm tissue. Using restriction endonucleases, the entire coding sequence for the Z4 gene is subcloned in the 3' to 5' orientation into an intermediate vector to form an antisense DNA sequence. The promoter region from a 10 kD zein protein, designated the Z10 promoter, is subcloned upstream from the antisense DNA sequence which includes the entire coding sequence for the Z4 gene to form an expression cassette. This expression cassette can then be subcloned into a vector suitable for transformation of plant cells.

In another preferred embodiment of the present invention, the promoter region from a 27 kD zein protein, designated the Z27 promoter, is subcloned upstream from the antisense DNA sequence.

In another preferred embodiment of the present invention, using restriction endonucleases, the entire coding sequence of the A20 gene encoding a 19 kD α -zein protein is subcloned in the 3' to 5' orientation into an intermediate vector to form an antisense DNA sequence. The Z10 promoter, or alternatively the Z27 promoter, is cloned upstream from the A20 antisense DNA sequence. Partial Z4 or A20 DNA sequences can also be cloned in an antisense 3' to 5' orientation downstream of the Z10 or Z27 promoter. Furthermore, it is contemplated that expression cassettes may be constructed which comprise the Z10 or Z27 promoter upstream of a partial or entire Z4 or A20 DNA sequences wherein said DNA sequences are subcloned downstream of the promoter in a 5' to 3' sense orientation.

15 2. Targeting Sequences

Additionally, expression cassettes can be constructed and employed to target the product of the preselected DNA sequence or segment to an intracellular compartment within plant cells or to direct a protein to the extracellular environment. This can generally be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of the preselected DNA sequence. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and can then be post-translationally removed. Transit peptides act by facilitating the transport of proteins through intracellular membranes, e.g., vacuole, vesicle, plastid and mitochondrial membranes, whereas signal peptides direct proteins through the extracellular membrane. By facilitating transport of the protein into compartments inside or outside the cell, these sequences can increase the accumulation of a particular gene product in a particular location. For example, see U.S. Patent No. 5,258,300.

When the expression cassette is to be introduced into a plant cell, the expression cassette can also optionally include 3' nontranslated plant regulatory DNA sequences that act as a signal to terminate transcription and allow for the polyadenylation of the resultant mRNA. The 3' nontranslated regulatory DNA sequence preferably includes from about 300 to 1,000 nucleotide base pairs and contains plant transcriptional and translational termination sequences. Preferred 3' elements are derived from those from the nopaline synthase gene of *Agrobacterium tumefaciens* (Bevan et al., 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens*, and the 3' end of the protease inhibitor I or II genes from potato or tomato, although other 3' elements known to those of skill in the art can also be employed. These 3' nontranslated regulatory sequences can be obtained as described in An (1987) or are already present in plasmids available from commercial sources such as Clontech, Palo Alto, California. The 3' nontranslated regulatory sequences can be operably linked to the 3' terminus of the preselected DNA sequence by standard methods.

In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible preselected DNA sequence or segment. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can 'select' for by chemical means, i.e., through the use of a selective agent (e.g., a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, i.e., by 'screening' (e.g., the R-locus trait). Of course, many examples of suitable marker genes are known to the art and can be employed in the practice of the invention.

Included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA; and proteins that are inserted or trapped in the cell wall (e.g., proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a polypeptide that becomes sequestered in the cell wall, and which polypeptide includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). The use of the maize HPRG (Stiefel et al., 1990) is preferred as this molecule is well characterized in terms of molecular biology, expression, and protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins (Keller et al., 1989) could be modified by the addition of an antigenic site to create a screenable marker.

Elements of the present disclosure are exemplified in detail through the use of particular marker genes. However in light of this disclosure, numerous other possible selectable and/or screenable marker genes will be apparent to those of skill in the art in addition to the one set forth herein below. Therefore, it will be understood that the following discussion is exemplary rather than exhaustive. In

light of the techniques disclosed herein and the general recombinant techniques which are known in the art, the present invention renders possible the introduction of any gene, including marker genes, into a recipient cell to generate a transformed plant cell, e.g., a monocot cell.

5 Possible selectable markers for use in connection with the present invention include, but are not limited to, a *neo* gene (Potrykus et al., 1985) which codes for kanamycin resistance and can be selected for using kanamycin, G418, and the like; a *bar* gene which codes for bialaphos resistance; a gene which encodes an altered EPSP synthase protein (Hinchee et al., 1988) thus conferring glyphosate
10 resistance; a nitrilase gene such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker et al., 1988); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonyleurea or other ALS-inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate-resistant DHFR gene (Thillet et al., 1988); a dalapon dehalogenase
15 gene that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan. Where a mutant EPSP synthase gene is employed, additional benefit may be realized through the incorporation of a suitable chloroplast transit peptide, CTP (European Patent Application 0 218 571, 1987).

20 An illustrative embodiment of a selectable marker gene capable of being used in systems to select transformants is the genes that encode the enzyme phosphinothricin acetyltransferase, such as the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *Streptomyces viridochromogenes* (U.S. Patent No. 5,550,318, which is incorporated by reference herein). The enzyme
25 phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami et al., 1986; Twell et al., 1989) causing rapid accumulation of ammonia and cell death. The success in using this selective system in conjunction with

monocots was particularly surprising because of the major difficulties which have been reported in transformation of cereals (Potrykus, 1989).

Screenable markers that may be employed include, but are not limited to, a β -glucuronidase or *uidA* gene (GUS) which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta et al., 1988); a β -lactamase gene (Sutcliffe, 1978), which encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a *xyIE* gene (Zukowsky et al., 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α -amylase gene (Ikuta et al., 1990); a tyrosinase gene (Katz et al., 1983) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily detectable compound melanin; a β -galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (*lux*) gene (Ow et al., 1986), which allows for bioluminescence detection; or an aequorin gene (Prasher et al., 1985), which may be employed in calcium-sensitive bioluminescence detection, or a green fluorescent protein gene (Niedz et al., 1995).

Genes from the maize R gene complex are contemplated to be particularly useful as screenable markers. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. Maize strains can have one, or as many as four, R alleles which combine to regulate pigmentation in a developmental and tissue specific manner. A gene from the R gene complex was applied to maize transformation, because the expression of this gene in transformed cells does not harm the cells. Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant alleles for genes encoding the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, A1, A2, Bz1 and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment

formation. Exemplary lines include Wisconsin 22 which contains the rg-Stadler allele and TR112, a K55 derivative which is r-g, b, Pl. Alternatively any genotype of maize can be utilized if the C1 and R alleles are introduced together.

It is further proposed that R gene regulatory regions may be employed
 5 in chimeric constructs in order to provide mechanisms for controlling the expression of chimeric genes. More diversity of phenotypic expression is known at the R locus than at any other locus (Coe et al., 1988). It is contemplated that regulatory regions obtained from regions 5' to the structural R gene would be valuable in directing the expression of genes, e.g., insect resistance, drought resistance, herbicide tolerance or
 10 other protein coding regions. For the purposes of the present invention, it is believed that any of the various R gene family members may be successfully employed (e.g., P, S, Lc, etc.). However, the most preferred will generally be Sn (particularly Sn:bol3). Sn is a dominant member of the R gene complex and is functionally similar to the R and B loci in that Sn controls the tissue specific deposition of
 15 anthocyanin pigments in certain seedling and plant cells, therefore, its phenotype is similar to R.

A further screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation
 20 counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It is also envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening.

5. Other Optional Sequences

25 An expression cassette of the invention can also further comprise plasmid DNA. Plasmid vectors include additional DNA sequences that provide for easy selection, amplification, and transformation of the expression cassette in prokaryotic and eukaryotic cells, e.g., pUC-derived vectors such as pUC8, pUC9, pUC18, pUC19, pUC23, pUC119, and pUC120, pSK-derived vectors, pGEM-

derived vectors, pSP-derived vectors, or pBS-derived vectors. The additional DNA sequences include origins of replication to provide for autonomous replication of the vector, additional selectable marker genes, preferably encoding antibiotic or herbicide resistance, unique multiple cloning sites providing for multiple sites to
 5 insert DNA sequences or genes encoded in the expression cassette, and sequences that enhance transformation of prokaryotic and eukaryotic cells.

Another vector that is useful for expression in both plant and prokaryotic cells is the binary Ti plasmid (as disclosed in Schilperoort et al., U.S. Patent No. 4,940,838) as exemplified by vector pGA582. This binary Ti plasmid
 10 vector has been previously characterized by An, cited *supra*, and is available from Dr. An. This binary Ti vector can be replicated in prokaryotic bacteria such as *E. coli* and *Agrobacterium*. The *Agrobacterium* plasmid vectors can be used to transfer the expression cassette to dicot plant cells, and under certain conditions to monocot cells, such as rice cells. The binary Ti vectors preferably include the nopaline T
 15 DNA right and left borders to provide for efficient plant cell transformation, a selectable marker gene, unique multiple cloning sites in the T border regions, the *colE1* replication of origin and a wide host range replicon. The binary Ti vectors carrying an expression cassette of the invention can be used to transform both prokaryotic and eukaryotic cells, but is preferably used to transform dicot plant cells.

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C. In Vitro Screening of Expression Cassettes

Once the expression cassette is constructed and subcloned into a suitable plasmid, it can be screened for the ability to substantially inhibit the translation of a mRNA coding for a seed storage protein by standard methods such as
 25 hybrid arrested translation. For example, for hybrid selection or arrested translation, a preselected antisense DNA sequence is subcloned into an SP6/T7 containing plasmids (as supplied by ProMega Corp.). For transformation of plants cells, suitable vectors include plasmids such as described herein. Typically, hybrid arrest translation is an *in vitro* assay which measures the inhibition of translation of a

mRNA encoding a particular seed storage protein. This screening method can also be used to select and identify preselected antisense DNA sequences that inhibit translation of a family or subfamily of zein protein genes. As a control, the corresponding sense expression cassette is introduced into plants and the phenotype
5 assayed.

II. DNA Delivery of the DNA Molecules into Host Cells

The present invention generally includes steps directed to introducing a preselected DNA sequence, such as a preselected cDNA, into a recipient cell to
10 create a transformed cell. The frequency of occurrence of cells taking up exogenous (foreign) DNA is believed to be low. Moreover, it is most likely that not all recipient cells receiving DNA segments or sequences will result in a transformed cell wherein the DNA is stably integrated into the plant genome and/or expressed. Some may show only initial and transient gene expression. However, certain cells from
15 virtually any dicot or monocot species may be stably transformed, and these cells regenerated into transgenic plants, through the application of the techniques disclosed herein.

The invention is directed to any plant species wherein the seed contains storage proteins that contain relatively low levels, or none, of at least one
20 essential amino acid. Cells of the plant tissue source are preferably embryogenic cells or cell-lines that can regenerate fertile transgenic plants and/or seeds. The cells can be derived from either monocotyledons or dicotyledons. Suitable examples of plant species include wheat, rice, *Arabidopsis*, tobacco, maize, soybean, and the like. The preferred cell type is a monocotyledon cell such as a maize cell, which may be in
25 a suspension cell culture or may be in an intact plant part, such as an immature embryo, or in a specialized plant tissue, such as callus, such as Type I or Type II callus.

Transformation of the cells of the plant tissue source can be conducted by any one of a number of methods known to those of skill in the art. Examples are:

The preferred method for dicot transformation is via infection of plant cells with *Agrobacterium tumefaciens* using the leaf-disk protocol (Horsch et al., 1985). Monocots such as *Zea mays* can be transformed via microprojectile bombardment of embryogenic callus tissue or immature embryos, or by electroporation following partial enzymatic degradation of the cell wall with a pectinase-containing enzyme (U.S. Patent No. 5,384,253; and U.S. Patent No. 5,472,869). For example, embryogenic cell lines derived from immature *Zea mays* embryos can be transformed by accelerated particle treatment as described by Gordon-Kamm et al. (1990) or U.S. Patent No. 5,489,520; U.S. Patent No. 5,538,877 and U.S. Patent No. 5,538,880, cited above. Excised immature embryos can also be used as the target for transformation prior to tissue culture induction, selection and regeneration as described in U.S. application Serial No. 08/112,245 and PCT publication WO 95/06128. Furthermore, methods for transformation of monocotyledonous plants utilizing *Agrobacterium tumefaciens* have been described by Hiei et al. (European Patent 0 604 662, 1994) and Saito et al. (European Patent 0 672 752, 1995).

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Methods such as microprojectile bombardment or electroporation are carried out with “naked” DNA where the expression cassette may be simply carried on any *E. coli*-derived plasmid cloning vector. In the case of viral vectors, it is desirable that the system retain replication functions, but lack functions for disease
5 induction.

The choice of plant tissue source for transformation will depend on the nature of the host plant and the transformation protocol. Useful tissue sources include callus, suspension culture cells, protoplasts, leaf segments, stem segments, tassels, pollen, embryos, hypocotyls, tuber segments, meristematic regions, and the
10 like. The tissue source is selected and transformed so that it retains the ability to regenerate whole, fertile plants following transformation, i.e., contains totipotent cells. Type I or Type II embryonic maize callus and immature embryos are preferred *Zea mays* tissue sources. Selection of tissue sources for transformation of monocots is described in detail in U.S. Application Serial No. 08/112,245 and PCT publication
15 WO 95/06128 (incorporated herein by reference).

The transformation is carried out under conditions directed to the plant tissue of choice. The plant cells or tissue are exposed to the DNA carrying the preselected DNA sequences for an effective period of time. This may range from a less-than-one-second pulse of electricity for electroporation to a 2-3 day co-
20 cultivation in the presence of plasmid-bearing *Agrobacterium* cells. Buffers and media used will also vary with the plant tissue source and transformation protocol. Many transformation protocols employ a feeder layer of suspended culture cells (tobacco or Black Mexican Sweet corn, for example) on the surface of solid media plates, separated by a sterile filter paper disk from the plant cells or tissues being
25 transformed.

A. Electroporation

Where one wishes to introduce DNA by means of electroporation, it is contemplated that the method of Krzyzek et al. (U.S. Patent No. 5,384,253, incorporated herein by reference) will be particularly advantageous. In this method, certain cell wall-degrading enzymes, such as pectin-degrading enzymes, are employed to render the target recipient cells more susceptible to transformation by electroporation than untreated cells. Alternatively, recipient cells can be made more susceptible to transformation, by mechanical wounding.

To effect transformation by electroporation, one may employ either friable tissues such as a suspension cell cultures, or embryogenic callus, or alternatively, one may transform immature embryos or other organized tissues directly. The cell walls of the preselected cells or organs can be partially degraded by exposing them to pectin-degrading enzymes (pectinases or pectolyases) or mechanically wounding them in a controlled manner. Such cells would then be receptive to DNA uptake by electroporation, which may be carried out at this stage, and transformed cells then identified by a suitable selection or screening protocol dependent on the nature of the newly incorporated DNA.

B. Microprojectile Bombardment

20 A further advantageous method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, microparticles may be coated with DNA and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

25 It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. In an illustrative embodiment, non-embryogenic BMS cells were bombarded with intact cells of the bacteria *E. coli* or *Agrobacterium tumefaciens* containing plasmids with either the β -glucoronidase or *bar* gene

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in a focus which express the exogenous gene product 48 hours post-bombardment often range from about 1 to 10 and average about 1 to 3.

In bombardment transformation, one may optimize the prebombardment culturing conditions and the bombardment parameters to yield the maximum numbers of stable transformants. Both the physical and biological parameters for bombardment are important in this technology. Physical factors are those that involve manipulating the DNA/microprojectile precipitate or those that affect the path and velocity of either the macro- or microprojectiles. Biological factors include all steps involved in manipulation of cells before and immediately after bombardment, the osmotic adjustment of target cells to help alleviate the trauma associated with bombardment, and also the nature of the transforming DNA, such as linearized DNA or intact supercoiled plasmid DNA. It is believed that pre-bombardment manipulations are especially important for successful transformation of immature embryos.

Accordingly, it is contemplated that one may wish to adjust various of the bombardment parameters in small scale studies to fully optimize the conditions. One may particularly wish to adjust physical parameters such as gap distance, flight distance, tissue distance, and helium pressure. One may also minimize the trauma reduction factors (TRFs) by modifying conditions which influence the physiological state of the recipient cells and which may therefore influence transformation and integration efficiencies. For example, the osmotic state, tissue hydration and the subculture stage or cell cycle of the recipient cells may be adjusted for optimum transformation. Results from such small scale optimization studies are disclosed herein and the execution of other routine adjustments will be known to those of skill in the art in light of the present disclosure.

III. Production and Characterization of Stable Transgenic Maize

After effecting delivery of a preselected DNA sequence to recipient cells by any of the methods discussed above, the next steps of the invention generally concern identifying the transformed cells for further culturing and plant regeneration.

- 5 As mentioned above, in order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible preselected DNA sequence. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

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A. Selection

- An exemplary embodiment of methods for identifying transformed cells involves exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been
15 transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

- To use the *bar*-bialaphos or the EPSPS-glyphosate selective system, bombarded tissue is cultured for about 0-28 days on nonselective medium and
20 subsequently transferred to medium containing from about 1-3 mg/l bialaphos or about 1-3 mM glyphosate, as appropriate. While ranges of about 1-3 mg/l bialaphos or about 1-3 mM glyphosate will typically be preferred, it is proposed that ranges of at least about 0.1-50 mg/l bialaphos or at least about 0.1-50 mM glyphosate will find utility in the practice of the invention. Tissue can be placed on any porous, inert,
25 solid or semi-solid support for bombardment, including but not limited to filters and solid culture medium. Bialaphos and glyphosate are provided as examples of agents suitable for selection of transformants, but the technique of this invention is not limited to them.

An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media. The R-locus is useful for selection of transformants from bombarded immature embryos. In a similar fashion, the introduction of the C1 and B genes will result in pigmented cells and/or tissues.

The enzyme luciferase is also useful as a screenable marker in the context of the present invention. In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter), by devices that enhance night vision, or by a highly light sensitive video camera, such as a photon counting camera. All of these assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time.

It is further contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as luciferase would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. In an illustrative embodiment embryogenic Type II callus of *Zea mays* L. was selected with sub-lethal levels of bialaphos. Slowly growing tissue was subsequently

screened for expression of the luciferase gene and transformants were identified. In this example, neither selection nor screening conditions employed were sufficient in and of themselves to identify transformants. Therefore it is proposed that combinations of selection and screening will enable one to identify transformants in a wider variety of cell and tissue types.

B. Regeneration and Seed Production

Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In an exemplary embodiment, MS and N6 media have been modified (see Table 1 of U.S. application Serial No.08/594,861, the disclosure of which is incorporated by reference herein) by including further substances such as growth regulators. A preferred growth regulator for such purposes is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or perhaps even picloram. Media improvement in these and like ways was found to facilitate the growth of cells at specific developmental stages. Tissue is preferably maintained on a basic media with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of manual selection, until the morphology of the tissue is suitable for regeneration, at least two weeks, then transferred to media conducive to maturation of embryoids. Cultures are transferred every two weeks on this medium. Shoot development will signal the time to transfer to medium lacking growth regulators.

The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, will then be allowed to mature into plants. Developing plantlets are transferred to soilless plant growth mix, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, about 600 ppm CO₂, and at about 25-250 microeinsteins m⁻²•s⁻¹ of light. Plants are preferably matured either in a growth chamber or greenhouse. Plants are

5 After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

Regenerated plants can be repeatedly crossed to inbred maize plants in order to introgress the preselected DNA sequence into the genome of the inbred maize plants. This process is referred to as backcross conversion. When a sufficient number of crosses to the recurrent inbred parent have been completed in order to produce a product of the backcross conversion process that is substantially isogenic with the recurrent inbred parent except for the presence of the introduced preselected DNA sequence, the plant is self-pollinated at least once in order to produce a homozygous backcross converted inbred containing the preselected DNA sequence. Progeny of these plants are true breeding and the weight percentage of a particular amino acid in a plant part, e.g., the seeds, or the amount of starch in these progeny are compared to the weight percentage of that amino acid or amount of starch in the recurrent parent inbred, in the field under a range of environmental conditions (see below). The determination of the weight percentage of an amino acid or amount of starch are well known in the art.

Alternatively, seed from transformed monocot plants regenerated from transformed tissue cultures is grown in the field and self-pollinated to generate true breeding plants.

Seed from the fertile transgenic plants is then evaluated for the presence and/or expression of the sense or antisense DNA sequence. Transgenic seed tissue can be analyzed for a substantial inhibition in the production of the seed storage protein using standard methods such as SDS polyacrylamide gel electrophoresis. A substantial inhibition of the production of the seed storage protein is a decrease in the weight percent of the seed storage protein, preferably of about 70-100% and more preferably about 80-100% over that normally present in a nontransformed seed. The weight percent of a seed storage protein or an amino acid is based upon the amount of that protein or amino acid present per total weight of all proteins or amino acids in the seed. The seed can also be evaluated for an increase in the weight percent of at least one amino acid essential in the diet of animals by standard methods. An increase in the weight percent of the target amino acid is preferably about 50-300%, and more preferably about 100-200%, over that normally present in the untransformed seed. While not in any way meant to limit the invention, the decrease in the expression in the target seed storage protein is generally accompanied by an increase in other proteins having amino acids essential in the diet of animals.

Once a transgenic seed expressing the sense or antisense DNA sequence and having an increase in the weight percent of the amino acid essential in the diet of animals is identified, the seed can be used to develop true breeding plants. The true breeding plants are used to develop a line of plants with an increase in the weight percent of an amino acid essential in the diet of animals as a dominant trait while still maintaining other desirable functional agronomic traits. Adding the trait of increasing the weight percent of an amino acid essential in the diet of animals to agronomically elite lines can be accomplished by back-crossing with this trait and with those without the trait and studying the pattern of inheritance in segregating

generations. Those plants expressing the target trait in a dominant fashion are preferably selected. Back-crossing is carried out by crossing the original fertile transgenic plants with a plant from an inbred line exhibiting desirable functional agronomic characteristics while not expressing the trait of an increased weight percent of the target amino acid. The resulting progeny are then crossed back to the parent not expressing the trait. The progeny from this cross will also segregate so that some of the progeny carry the trait and some do not. This back-crossing is repeated until the inbred line with the desirable functional agronomic traits, but without the trait of an increase in the weight percent of an amino acid essential in the diet of animals, which is expressed in a dominant fashion.

Subsequent to back-crossing, the new transgenic plants are evaluated for an increase in the weight percent of an amino acid essential in the diet of animals as well as for a battery of functional agronomic characteristics. These other functional agronomic characteristics include kernel hardness, yield, resistance to disease and insect pests, drought resistance, and herbicide resistance.

Plants that may be improved by these methods include but are not limited to processed plants (canola, potatoes, tomatoes, lupins, sunflower and cottonseed), forage plants (alfalfa, clover and fescue), and the grains (maize, wheat, barley, oats, rice, sorghum, millet and rye). The plants or plant parts may be used directly as feed or food or the amino acid(s) may be extracted for use as a feed or food additive.

C. Determination of Stably Transformed Plant Tissues

To confirm the presence of the preselected DNA sequence in the regenerating plants, or seeds or progeny derived from the regenerated plant, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting and PCR; "biochemical" assays, such as detecting the presence of a protein product, e.g., by immunological means (ELISAs and Western blots) or by

enzymatic function; plant part assays, such as leaf, seed or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA may only be expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR techniques may also be used for detection and quantitation of RNA produced from introduced preselected DNA segments. In this application of PCR it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and will only demonstrate the presence or absence of an RNA species.

While Southern blotting and PCR may be used to detect the preselected DNA segment in question, they do not provide information as to whether the preselected DNA segment is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced preselected DNA sequences or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focussing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their

presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as Western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of preselected DNA segments encoding storage proteins which change amino acid composition and may be detected by amino acid analysis.

IV. Increasing the Weight Percent of at Least One Amino Acid Essential to the Diet of Animals.

The present invention is directed to increasing the amount of an amino acid essential to the diet of animals in a transgenic plant or seed over that normally present in the corresponding nontransformed (nontransgenic) plant or its seed. Plant cells are stably transformed with a preselected DNA sequence that encodes a RNA molecule having substantial identity (sense), or complementarity (antisense), to a mRNA coding for a seed storage protein, preferably a seed storage protein which is deficient in at least one amino acid essential in the diet of animals. The transformed cells are used to regenerate fertile transgenic plants and seeds. The antisense, or sense, RNA sequence is expressed in the seeds in an amount effective to inhibit the production of the seed storage protein. The decrease in the seed storage protein deficient in the essential amino acid results in an increase in the weight

percent of other amino acids, preferably essential amino acids, present in other proteins in the transgenic seed over that normally present in the nontransformed seed.

In a preferred embodiment, a maize cell line is transformed with an expression vector comprising a preselected DNA sequence coding for a RNA molecule substantially identical, or complementary, to all or a portion of a mRNA coding for a 19 kD or 22 kD α -zein protein operably linked to a promoter for a 10 kD zein protein. Another preferred embodiment includes linking the preselected DNA sequence to the Z27 promoter. The expression vector preferably further comprises at least one selectable marker gene. The maize cell line is transformed by biolistic transformation and transformants are initially selected by growth in the presence of an agent which is present at levels which inhibit the growth of the corresponding nontransformed cells. Transformants are further characterized for the presence or expression of the preselected DNA sequence by polymerase chain reaction (PCR) or reverse transcriptase (RT-PCR) analysis. Transformed maize cell lines having the preselected DNA sequence are used to regenerate fertile transgenic plants by the method as described in PCT publication WO 95/06128. The fertile transgenic plants are self-pollinated or crossed to a second plant variety, and the transgenic seeds are characterized for the inhibition of production of a 19 kD or 22 kD α -zein protein by quantitative Western blot, or SDS-PAGE, and for an increase in the weight percent of an amino acid essential to the diet of animals, such as lysine.

In an alternative embodiment, the present invention is directed to increasing the weight percent of an amino acid essential in the diet of animals in a plant or seed by stably transforming the cells of a plant tissue source with at least two different preselected DNA sequences. The first preselected DNA sequence comprises a preselected DNA sequence coding a RNA molecule substantially identical, or complementary, to a mRNA for a seed storage protein, preferably a seed storage protein which is deficient in at least one amino acid essential to the diet of animals. The second preselected DNA sequence encodes a polypeptide comprising at least one amino acid essential to the diet of animals. The expression cassettes

comprising one or both of the preselected DNA sequences can optionally comprise a selectable marker gene and, optionally, a reporter gene. Each preselected DNA sequence may comprise a different selectable marker gene so that transformants containing both preselected DNA sequences can be readily selected.

- 5 The cells of plant tissue source, as well as the methods of transformation described previously, can be employed in co-transformation. Co-transformation can be conducted sequentially, that is, the cells of plant tissue source can be transformed with the first preselected DNA sequence and transformants selected. The transformants can then be transformed with the second preselected
- 10 DNA sequence and transformants having both preselected DNA sequences can be selected. Typically, the initial selection is based upon the trait expressed by the selectable marker gene or genes. Co-transformation can also be conducted in one step, that is, the cells of the plant tissue source can be transformed with both preselected DNA sequences at once, e.g., by electroporation or biolistic
- 15 transformation. Alternatively, two plants can be crossed. The genome of one of the plants comprises the first preselected DNA sequence and the genome of the other plant in the cross comprises the second preselected DNA sequence.

- Transformants containing both preselected DNA sequences are further characterized for the presence and/or expression of the first preselected DNA
- 20 sequence and the second preselected DNA sequence by standard methods, such as PCR or RT-PCR, Southern blot hybridization, SDS-PAGE and quantitative Western blot. Transformants having both introduced sequences are used to generate fertile transgenic plants and seeds therefrom as described previously.

- The transgenic seeds are then characterized for the presence and/or
- 25 expression of both preselected DNA sequences. Expression of the first preselected DNA sequence can be detected and quantitated by examining the seeds for a substantial inhibition of the production of a seed storage protein deficient in an amino acid essential in the diet of animals. Expression of the second preselected DNA sequence can be detected and quantitated by quantitative Western blot for the

plant protein comprising at least one amino acid essential in the diet of animals and/or by an increase in the weight percent of an amino acid essential in the diet of animals, such as lysine or methionine, as compared to an untransformed seed.

In a preferred embodiment, a maize cell line is co-transformed with a first preselected DNA sequence coding for a RNA molecule substantially identical, or complementary, to all or a portion of a mRNA coding for a 19 kD or 22 kD α -zein protein, and a second preselected DNA sequence coding for a 10 kD zein protein. The 19 kD or 22 kD α -zein protein is preferably deficient in at least one amino acid essential in the diet of animals, such as lysine, methionine or tryptophan. The 10 kD zein protein preferably comprises at least one amino acid essential in the diet of animals, such as methionine. The isolated, purified DNA molecule comprising the first preselected DNA sequence also preferably comprises a selectable marker gene or a reporter gene, such as GUS. The second preselected DNA sequence may contain a second selectable marker gene, such as glyphosate resistant EPSPS.

In a further embodiment of the present invention, maize is cotransformed with a first preselected sense DNA sequence coding for a RNA molecule which is identical, or complementary, to the 19 kD or 22 kD α -zein mRNA and a second preselected DNA sequence encoding the synthetic protein MB1. Alternatively, the second preselected DNA sequence encodes a 27 kD zein protein. Thus, it is contemplated that genes encoding other synthetic or naturally occurring proteins comprising at least one amino acid essential in the diet of animals may be substituted for MB1. Even more preferably, maize is cotransformed with a first preselected sense DNA sequence coding for a RNA molecule which is identical, or complementary, to the 19 kD or 22 kD α -zein mRNA, a second preselected DNA sequence encoding the synthetic protein MB1, and a third preselected DNA sequence encoding a 27 kD zein protein.

Transformants having both preselected DNA sequences are used to generate fertile transgenic plants and seeds. The transgenic seeds are characterized by a substantial inhibition of the production of a 19 kD or 22 kD α -zein protein,

5 in the diet of animals can be expressed as a dominant trait while still maintaining functional agronomic qualities, as described hereinabove.

V. Method to Increase Starch Content of a Plant Seed

The invention also provides for an increase in the weight percent of starch in a plant and/or seed. The method comprises stably transforming the cells of a plant tissue with a first preselected DNA sequence coding for an RNA molecule substantially homologous or complementary to all or a portion of a mRNA coding for at least one seed storage protein. While not in any way meant to limit the invention, it is believed that a decrease in the expression of seed storage protein in the seed results in an increase in the weight percent of the starch in the seed. The preselected DNA sequence is preferably operably linked to a promoter functional in a plant and/or seed. Transformed cells are used to regenerate fertile transgenic plants and/or seeds. The transgenic seeds are characterized for expression of the preselected DNA sequence by examining the seed for a substantial inhibition of the production of at least one seed storage protein and for an increase in the weight percent of starch over that normally present in an untransformed seed.

The first preselected DNA sequence can be derived from a DNA sequence encoding at least one plant seed storage protein. Plant seed storage proteins include the zein proteins of maize such as the α -, β -, γ -, or δ -zein proteins.

25 While not in any way meant to limit the invention, it is believed that a decrease in the expression of seed storage protein in the seed results in an increase in the weight percent of the starch in the seed. Preferably, the presence of the first preselected DNA sequence results in a substantial inhibition of at least one seed storage protein, and more preferably results in the inhibition of the α -zein proteins. The preparation

of said first DNA sequence as well as its linkage to suitable promoters can be accomplished as described hereinabove. Cells of plant tissue can be transformed as described above, and transformants selected. Transformants are used to generate fertile transgenic plants and seeds.

Transgenic seeds are characterized by an increase in the weight percent of starch in the seed over that present in the untransformed seed. The weight percent of the starch content in the seed can be determined by enzymatic hydrolysis and glucose determination. The weight percent of starch is calculated by comparing the weight of the starch in the seed compared to the total weight of the seed. An increase in the weight percent of the starch in the transgenic seed is preferably about 1 to 10%, and more preferably about 3 to 8%, and even more preferably about 5 to 7%, over that in the non-transformed seed.

Transgenic seeds with an increase in the weight percent of starch can be used to develop true breeding plants expressing this trait in a dominant fashion while still maintaining functional agronomic traits as described previously.

Reduction of α -zein levels in corn kernels may also increase the degree of starch recovery from operations such as wet-milling of grain as α -zeins constitute the major portion of the proteinaceous matrix which surrounds starch granules in the kernel (Lopes and Larkins, 1993). A reduction in the amount of these hydrophobic proteins could facilitate recovery of starch grains. This is of particular significance for specialty starches, such as that obtained from high-amylose corn or waxy corn, because those starches are of much higher value than that obtained from No. 2 yellow dent corn. An increase in starch yield, i.e., the percent of starch present in the kernel which may be recovered by wet milling, is preferably about 1% to 20%, more preferably about 3% to 15%, and even more preferably about 6% to 12%, greater in grain from plants containing the preselected DNA sequence over grain from plants which do not contain the preselected DNA sequence.

VI. A Method for Inhibiting the Expression of a Family or Subfamily of Seed Storage Proteins.

The invention also provides a method for inhibiting the expression of a family, or subfamily, of seed storage proteins. Seed storage proteins such as the maize zein proteins are encoded by multi-gene families. The multi-gene families corresponding to zein proteins have different molecular weights: α -zein proteins include proteins with molecular weights of 19 kD and 22 kD; β -zein proteins include proteins with a molecular weight of 14 kD; γ -zein proteins include proteins with molecular weights of about 27 kD and 16 kD; and δ -zein proteins include proteins with molecular weights of about 10 kD. Each family can have several subfamilies. For example, the subfamilies for α -zein proteins are determined on the basis of sequence homology to cDNA clones A20, A30, B49, B59, or B36 as described by Messing et al., cited *supra*., or the Z4 cDNA clone encoding the 22 kD α -zein. Typically, members of the same subfamily share about 90% to 100% amino acid sequence homology and members of different subfamilies share about 60% to 80% amino acid sequence homology.

The examination of the amino acid sequence for the α -zein subfamilies has identified four functional subdomains and regions of shared amino acid homology in these functional subdomains as shown in Figure 1. These regions of amino acid sequence homology can be used to analyze amino acid sequences from other subfamilies and families of zein proteins for homology. In addition, these regions can be used to select DNA sequences that encode a RNA molecule that can inhibit production of a family or a subfamily of the zein proteins. An antisense RNA sequence than can inhibit production of a family or subfamily of zein proteins is preferably a sequence that is substantially complementary to a portion of a mRNA sequence that is substantially homologous between all members of the subfamily or family of the zein proteins. Alternatively, it is contemplated that preselected sense DNA sequences may be used to suppress the synthesis of a family or a subfamily of zein.

For example, as shown in Figure 1, the A20, A30, and B49 subfamilies share amino acid sequence homology in the signal peptide region and amino terminal region of the proteins. An antisense DNA sequence encoding these regions of the zein protein can encode a RNA molecule that can inhibit expression for a family of zein proteins. The antisense DNA sequence encoding these regions can be selected based on the amino acid sequence homology in these regions and can be used to inhibit expression of more than one subfamily of a family of the zein proteins.

The domain containing the tandem repeats of 20 amino acids has the greatest variability in amino acid sequence and size. There are insertions and deletions in this region when the sequence of different subfamilies are compared. A preselected antisense DNA sequence encoding this region of the α -zein protein can be employed to express a RNA molecule that can inhibit the expression of a subfamily of zein proteins. The preselected antisense DNA sequence from this region of the zein protein is substantially homologous within a subfamily but is not substantially homologous between subfamilies.

The preselected antisense DNA sequence is obtained by restriction endonuclease digestion of a cDNA or genomic clone coding for a seed storage protein. The preselected antisense DNA sequence is linked to a promoter to form an antisense expression cassette to determine the capacity of the antisense DNA sequence to inhibit translation of a family or subfamily of seed storage proteins. A standard assay such as hybrid arrested translation may be employed. The preselected antisense DNA sequence results in substantial inhibition of translation of cDNA clones from several families such as A20, Z4, A30, and/or B49. The preselected antisense DNA sequence can inhibit a family of zein proteins. The preselected antisense DNA sequence substantially inhibits translation of cDNA clones or genomic clones within a subfamily and the preselected antisense DNA sequence can be used to inhibit expression of a subfamily of zein proteins. The preselected antisense DNA sequence is used to stably transform plant cells as described

hereinabove. Sense DNA sequences may also be used. Fertile transgenic plants and seeds are generated from the transformed cells.

The transgenic seeds are characterized for expression of the preselected antisense DNA sequence by evaluating inhibition of production of two or more members of a family or subfamily of zein proteins by using techniques such as quantitative Western blot.

In a preferred embodiment, the preferred antisense DNA sequence coding for a RNA molecule substantially complementary to a mRNA coding for the tandem repeat region of domain 3 of an α -zein protein in A20 subfamily is combined with a Z10 promoter. The expression cassette comprising the preselected antisense DNA sequence can also comprise one or more selectable marker genes. The preselected antisense DNA sequence is stably transformed into a maize cell line and transformants are selected. Transformed cells are used to generate fertile transgenic plants and seeds. The transgenic seeds are evaluated for expression of the preselected antisense DNA sequence by confirming substantial inhibition in the production of the A20 subfamily of α -zein proteins by quantitative Western blot.

VII. Method for Increasing the Production of a Preselected Polypeptide in Seeds

The invention further provides for an increase in the expression of a particular polypeptide in plants and/or seeds. The method involves stably transforming cells with a first preselected DNA sequence to suppress synthesis of a seed storage protein deficient in an essential amino acid and a second preselected DNA sequence coding for a polypeptide, such as an enzyme or a seed storage protein. While not in any way meant to limit the invention, it is believed that a substantial inhibition of production of at least one seed storage protein is accompanied by an increase in the capacity of the plant cell and/or seed to produce other proteins. Transformed cells having both first and second preselected DNA sequences are obtained and used to generate fertile transgenic plants and/or seeds.

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The transgenic seeds are characterized by expression of the first and second preselected DNA sequences. Expression of the first preselected DNA sequence is evaluated by measuring a substantial inhibition in the production of at least one seed storage protein. Expression of the second preselected DNA sequence is evaluated by detecting the preselected polypeptide using standard phenotypic or genotypic methods, such as quantitative Western blot. An increase in the expression of a polypeptide can be determined by comparing the weight percent of the protein produced in plants or seeds transformed with the second preselected DNA sequence. The expression of the polypeptide is preferably increased about 2- to 100-fold, and more preferably about 5- to 30-fold, over that in a plant and/or seed only transformed with the second preselected DNA sequence.

The invention will be further described by the following examples.

EXAMPLE 1

Construction of Plasmid Containing Antisense DNA Constructs

Antisense expression cassettes were obtained by using sequences from cDNA clones encoding zein proteins. The cDNA clones were prepared by standard methods, described previously by Geraghty et al. (1982) and Hu et al. (1982), which are hereby incorporated by reference. The cDNA clone A20 encodes an α -zein protein of the 19 kD size class of the Z1A subfamily of zein genes. Another cDNA clone designated Z4 encodes an α -zein of the 22 kD size class of the Z1B family of genes. The Z1A and Z1B subfamilies and their characteristics are shown in Table I.

TABLE I

Prolamin Fraction of Maize (Alcohol Soluble) Zein Multigene Family

Subfamily	z1				z2		
	z1A	z1B	(non-reducing conditions)		(reducing conditions)		
Representing cDNA clone	A20	A30	z1C	z1D	z2A (ASC)	z2B	z2C
Mr x 100	mostly 19	mostly 19 some 22	mostly 22 some 19	mostly 19	27	15	10
Locus	4L, 7S, 10L	4L, 7S	4L	?	?	?	?
Predominant amino acid	glutamine	glutamine	glutamine	glutamine	proline	cysteine	methionine
Timing of expression	ca 12 dap	ca 12 dap	ca 18 dap	ca 12 dap	ca 18 dap	ca 18 dap	ca 18 dap
Transacting mutants	o2+.o7+++	o2+.o7++	o2+++o7++	o2+.o7++	o2+.o7+	o2+.o7++	o2+.o7++
(06+++f12+.Mc+)	De*-B30+	De*-B30+	De*-B30+++	De*-B30+	De*-B30+	De*-B30+	De*-B30+
No. of genes	< 25	< 20	< 15	< 5	2	2	?

+ reduced synthesis

++ increasingly reduced synthesis

+++ strongly reduced synthesis

Antisense expression cassettes comprising the complete cDNA sequence for clones A20 and Z4, as well as portions of those sequences, were generated. The portions of each sequence were selected by examining the sequence of the 19 kD and 22 kD α -zein proteins. As shown in Figure 1, the primary sequence of the polypeptides can be divided into four domains, as described by Messing et al. (1983). Domain I contains the highly conserved 21 amino acid signal peptide that is cleaved during cotranslational transport of zein proteins into the lumen of the endoplasmic reticulum. Domains II and IV are the N-terminal and C-terminal regions, respectively, of the mature zein proteins. Domain III represents the major source of sequence homology between subfamilies as it contains 9-10 tandem repeats of sequence encoding a 20 amino acid sequence. The number of repeats present in Domain III determines the size of the α -zein protein (19 kD or 22 kD). Typically, individual members within a subfamily share 90-100% sequence homology and while the sequence homology between subfamilies ranges from about 65-85%.

All antisense plasmids for *in vitro* system analysis were constructed by standard recombinant techniques as detailed below, using the transcriptional vectors pSP72 and pSP73 (Promega, Madison, WI). These transcription vectors are 2.46 kb circular plasmids, containing 103 bp of polylinker sequence inserted between convergent T7 and SP6 transcriptional promoters. The two transcription vectors differ in the orientation of the polylinker with respect to the promoters. Antisense plasmids, complementary to all or portions of the cDNA clones A20 and Z4, were constructed as described below.

The RNA sequence for A20 (SEQ ID NO:1) and the DNA sequence for Z4 (SEQ ID NO:2) zein are shown in Figures 2 and 3, respectively. The relevant A20 and Z4 genes and gene fragments used in antisense constructs are shown in Table II.

TABLE II

	Antisense Construct <u>Designation</u>	Restriction <u>Enzymes</u>	Insert <u>Size</u>
5	SP20 ent	BalI/EcoRI	711
	SP20R3'	BalI/PstI	488
10	SP20R	PstI/PstI	262
	SP20P	BalI/EcoRI	863
	SP20P5'	AccI/EcoRI	458
	SPZ4ent	SacI/BamHI	960
	SPZ4R3'	XbaI/BamHI	713
15	SPZ4R5'	BamHI/DdeI	246
	SPZ10ent	EcoRI	640

All restriction and modification enzymes and buffers were obtained from New England Biolabs, Inc. (Beverly, MA), unless otherwise noted, and used according to the manufacturer's specifications. All insert fragments were gel isolated and purified by the GeneClean method (BIO 101, Vista, CA), and all vectors were treated with calf intestinal phosphatase (Boehringer-Mannheim Corporation, Indianapolis, IN), then gel isolated on low melting point agarose before addition to the ligation reactions.

Antisense constructs encoding all or a portion of the cDNA clones from A20 and Z4 were prepared as follows:

SP20ENT: The parent plasmid pUC12/A20, containing the entire mature coding and 3' nontranslated sequence (nts) from the A20 cDNA clone (the RNA sequence of A20 is shown in Figure 2), was digested at the EcoRI site (nt 175) and the BalI site (nt 886) to generate a 711 nt fragment containing the entire sequence except for 55 bp of 3' nts. The fragment was ligated into pSP72 which had been digested with

EcoRI and PvuII, resulting in 3' to 5' antisense orientation of the gene with respect to the SP6 promoter.

SP20R3': A 488 bp fragment, containing the sequence encoding the mid-repeat region through the 3' nts A20 from the Pst I site at nt 298 to the Ball site at nt 886, was isolated from the parent plasmid p1020R3' prepared as in Example 2. The fragment was obtained by digesting p1020R3' with KpnI and HindIII, and after isolation the fragment was ligated into pSP72 that had been digested with these enzymes also. The gene fragment was therefore oriented 5' to 3' with respect to the SP6 promoter.

SP20R: A 262 bp fragment, from nt 398 to nt 660 was obtained by digesting pUC12/A20 with PstI. The purified fragment was ligated into pSP72 digested with PstI to make pSP20R, containing the sequence encoding the mid-repeat region of A20 in the 3' to 5' orientation with respect to the SP6 promoter.

SP20P: The 5' end of the A20 transcription unit was reconstructed by PCR amplification of a fragment containing 5' nts and encoding the signal peptide through the mid-repeat region, since the 5' nts and signal peptide sequence was not contained in the pUC12/A20 clone. The primers used in the amplification are designated A20P5'.2 (SEQ ID NO:3) and A20P3' (SEQ ID NO:4). The fragment was amplified from genomic DNA isolated from leaf tissue from the maize inbred line A654, and contained 458 bp of A20 cDNA sequence, from nt 58 to nt 490.

The conditions for PCR are detailed below; all reactions were carried out in a Biosycler™ oven (Bios Corporation). Each reaction contained 10 µl of 10X PCR reaction buffer, 10 µl of 20 mM MgCl₂, 10 µl of 2 mM dNTPs, 10 µl of each primer (stock 2.5 ml) and 0.5 µl (2.5 U) of Taq polymerase (Perkin-Elmer Cetus), for a total of 100.5 µl/reaction. An annealing temperature of 56°C was used, and a

total of 30 cycles were performed, including the first three cycles with extended incubation at the 94°C denaturing temperature. Parameters for the first three amplification cycles were as follows: 60 seconds at 94°C, 30 seconds at the annealing temperature of 56°C, and 30 seconds at the synthesis temperature of 72°C. For the remaining 27 cycles, the parameters were as follows: after bringing the reactions to 94°C, 15 seconds at this temperature, then 15 seconds at 56°C, followed by 15 seconds at 72°C.

The 458 bp product was designed to add a 5' EcoR1 site, and included an endogenous 3' AccI site. After digestion with these enzymes, the amplified fragment was ligated into pSP20ENT also digested with these enzymes, replacing a 320 bp fragment containing the shorter 5' end fragment of A20 from pSP20ENT. After reconstruction, the gene was approximately 860 bp long, and contained approximately 55 nt of 5' nts, the sequence encoding the signal peptide, and the entire coding sequence as well as 3' nts. The reconstructed gene is oriented 3' to 5' with respect to the SP6 promoter.

SP20P5': The 5' end of the A20 gene, after PCR amplification and digestion with EcoRI and AccI as described above, was cloned into pSP72 to generate pSP20P5'. This construct contains 458 nt of A20 sequence, including 55 nt of 5' nontranslated sequence and 403 nt of coding sequence, which includes approximately the N terminal half of the coding sequence. The inserted sequence is oriented from 3' to 5' with respect to the SP6 promoter.

SPZ4ENT: Essentially, the entire Z4 transcription unit is contained in this clone, with a total insert size of 960 nt. The gene was reconstructed from two Z4 subclones, pSPZ4R3' and pSPZ45', which are described below. The parent vector was pSPZ4R3', containing 713 nt of mid-repeat to 3' nts sequence, from nt 630 to nt 1341 of the Z4 sequence (the DNA sequence of Z4 is shown in Figure 3). The 5'

end of the Z4 sequence was released by digestion with SacI (which cleaves the polylinker sequence outside the inserted gene) and BamHI, and the insert containing the 5' sequence from pSPZ45', obtained by SacI (which also cleaves the polylinker sequence) an dBamHI digestion, was ligated to the linearized pSPZ4R3', resulting in reconstitution of the intact Z4 transcription unit.

SPZ4R3': A 713 nt insert fragment, containing the mid-repeat region to the 3' noncoding sequence, was isolated after digestion with BamHI (nt 630) and XbaI (nt 1341). The fragment was ligated into pSP72 digested with the same enzymes, resulting in orientation of the gene fragment in 3' to 5' direction with respect to the SP6 promoter.

SPZ45': A 247 nt fragment containing 76 nt of 5' noncoding sequence, the signal peptide sequence, and approximately 100 nt of mature protein coding sequence was cloned into pSP72. After digestion with DdeI, the DNA was Klenow treated to create blunt ends, then digested with BamHI to release the desired fragment. The fragment was ligated into pSP72 digested with EcoRV and BamHI, resulting in 3' to 5' orientation of the gene fragment with respect to the SP6 promoter.

SPZ10ENT: A 670 nt fragment containing the entire Z10 transcriptional unit was isolated from the 10 kD zein cDNA clone p10kZ-1 by digestion with EcoRI (the sequence of the 10 kD zein gene can be found in Kiriha et al., 1988). After digestion of pSP72 with EcoRI also, the insert was ligated with the vector to produce pSPZ10ENT, a circular plasmid of 3.16 kb. Clones were obtained containing both orientations, and the clone used in the hybrid arrest studies contained the 10 kD transcription unit oriented 3' to 5' with respect to the SP6 promoter.

EXAMPLE 2

Construction of Plasmids Containing an Antisense DNA Sequence for Use in Maize Transformation

A set of antisense plasmids was constructed for expression in maize, using entire or portions of the Z4 and A20 sequence as detailed in Example 1, above. The antisense constructs were combined with a promoter functional in plant endosperm tissue to form a DNA sequence that can be expressed in a plant seed.

10 Vector Construction

The plasmids p10B and p10X were constructed from pZ10nos3'. The construct pZ10nos3' contains 1137 bp of the Z10 promoter from a gene encoding a 10 kD zein promoter upstream of a short polylinker, which is adjacent to the nos poly A 3' element. The vectors p10X and p10B were created by digestion of pZ10nos3' with BamHI, Klenow treatment to blunt the BamHI site, then ligation with a polylinker insert, resulting in clones containing both orientations of the polylinker with respect to the Z10 promoter. The polylinker fragment was obtained by digesting pSP73 with BglIII and XhoI, followed by Klenow treatment then ligation with the prepared pZ10nos3' vector. The p10X version contains the polylinker oriented with the XhoI site proximal to the Z10 promoter, while the p10B version contains the polylinker oriented with the BglIII site proximal to the Z10 promoter. Both plasmids are circular plasmids of approximately 4.65 kb. Antisense DNA expression constructs, prepared as described in Example 1 were combined with a promoter functional in a plant seed utilizing the p10B and p10X plasmids, as described below.

1020ENT: A 725 nt insert fragment containing the mature A20 coding and 3'noncoding sequence (see Example 1, SP20ENT section), and including some polylinker sequence, was obtained by digestion of SP20ENT with ClaI (cuts in the polylinker sequence) and XhoI. The vector, p10X, was prepared by digestion with

ClaI and XhoI also, then the insert and vector were ligated, generating p1020ENT, which contains the A20 sequence inserted 3' to 5' with respect to the Z10 promoter.

1020R3': A 488 nt insert fragment, containing the mid-repeat to the 3' noncoding sequence of A20, was isolated from the clone pUC12/A20. The insert contains sequence from the PstI site at nt 398 and continues to the Ball site at nt 886. The insert was obtained by digestion of pUC12/A20 with Hind III which cuts outside the A20 sequence, then partial digestion with PstI (digestion only at the nt 398 PstI site), followed by gel isolation of the desired fragment of 740 nt. After purification, the HindIII/PstI fragment was digested with Ball, which removed approximately 252 nt from the 3' end to generate a 488 nt fragment with PstI/Ball ends. This fragment was ligated into p10B which had been cut with SmaI and PstI, resulting in insertion of the A20R3' fragment in the 3' to 5' orientation with respect to the Z10 promoter.

1020R: A 262 nt insert fragment, containing the mid-repeat region from A20 (as in SP20R from Example 1), was obtained by digestion of pUC12/A20 with PstI. The vector p10X was also digested with PstI and, after ligation, clones were obtained with both orientations of the fragment with respect to the Z10 promoter. An asymmetrical AccI site within the insert was used to select clones containing the fragment in the desired antisense orientation.

pDPG380: The 863 nt insert fragment containing the reconstructed A20 gene (as described for pSP20P above) was obtained by digesting pSP20P with XhoI and BglII (both of which cut in the polylinker), then ligating the fragment into p10X that had been digested with XhoI and BamHI. This resulted in a 3' to 5' orientation of the reconstructed A20 gene with respect to the Z10 promoter.

pDPG340: A 875 nt fragment, containing the entire Z4 gene as described above for pSPZ4ENT, was obtained by digestion of pSPZ4ENT with HindIII, Klenow treatment, then digestion with SalI. These enzymes cut in the polylinker sequence outside the gene in pSPZ4ENT. The vector, p10X, was digested with NcoI, Klenow treated, then digested with XhoI before ligation with the insert fragment. The resulting clone contained the gene in 3' to 5' orientation with respect to the Z10 promoter.

10Z4R3': An insert of approximately 750 nt, consisting of the Z4 mid-repeat through the 3' noncoding (as described in Example 1 for pSPZ4R3') was obtained by digesting pSPZ4R3' with SacI and SalI, which cut in the polylinker sequence. The vector, p10X, was digested with SalI and XhoI, and since XhoI and SalI create compatible ends, this resulted in directional cloning of the Z4R insert in the 3' to 5' orientation with respect to the Z10 promoter.

10Z45': An intermediate vector 119Z45', containing the Z45' sequence insert (see SPZ45' construction, Example 1) was first constructed using the pUC119 backbone (Sambrook et al., 1989).

The final construct, 10Z45RN, was constructed by moving the Z45' insert from 119Z45' into the p10B vector. First, 119Z45' was digested with BamHI and PstI, releasing a 270 bp fragment. The vector, p10B, was prepared by digestion with BamHI and PstI, and then the vector and insert were ligated to produce p10Z45', containing the Z45' insert in the antisense orientation with respect to the Z10 promoter.

pDPG530 and pDPG531: pDRG530 and pDRG531 were made by cutting a fragment of approximately 960 bp from SPZ4Ent and filling in the ends. The vector was a Z27promoter::Nos 3' region construct in pBSK(-) which contained a unique

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In Vitro Method for Screening Antisense Containing DNA Sequences

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Transcription reactions. After thawing all reagents at room temperature, master transcription mixes were prepared, excluding template DNA. This resulted in greater yield uniformity of the reactions. For each reaction, the following components were added to 5 µl of template DNA at 1 µg/µl in RNase-free water:

- 5 20 µl of 54 transcription buffer, 10 µl at 0.1 M DTT, 2.5 µl of recombinant RNasin (an RNase inhibitor supplied at 40 U/µl), 20 µl of 10 mM rNTP mix, 2.5 µl of SP6 or T7 (20 U/µl), and 45 µl of RNase-free water. Reactions were incubated at 37°C for two hours before template removal. Templates were removed by digestion with RQ1 DNase (1 U/µl), 5.0 µl of enzyme was added to the transcription reactions,
- 10 which were then incubated at 37°C for 15 minutes before extraction and precipitation of the transcript. Extraction, precipitation and washes were performed as described above for template preparation.

- Transcript yield was determined by absorbance readings at 260 nm, and intactness of the preparations was determined by gel analysis, either native or
- 15 denaturing. Although native gels occasionally showed bands of anomalous mobility, generally transcript preparations exhibited a roughly linear relationship between the expected transcript size and their mobility on native gels.

- Annealing of Transcripts for Hybrid Formation. Before translation, transcripts were
- 20 allowed to anneal under controlled temperature and salt conditions, using constant molar ratios of sense to antisense transcript. Conditions for annealing were as follows: 10 mM Tris, pH 7.5, 100 mM NaCl, RNA(s), and RNase-free water to bring the total volume to 20 µl. The amount of RNA added was based on a 4:1 molar ratio of antisense to sense transcript, with 4 µg of sense transcript in each
- 25 reaction, and a variable µg amount of antisense transcript added to maintain the 4:1 molar ratio.

Before annealing, all transcripts were heated to 65°C, then kept at 0°C to reduce potential formation of intramolecular secondary structures which would reduce the efficiency of duplex formation. After annealing for 45 minutes at

45°C, the reaction was split in half, so that 10 µl of the reaction could be translated *in vitro*, and the remaining 10 µl was analyzed on 1.2% agarose gels to determine the extent of hybrid formation in each sample. Although some anomalies in mobility were seen that were probably due to intramolecular interaction, this method was generally useful for analyzing the extent of duplex formation between two transcripts, and correlated well with the hybrid translation results.

In Vitro Translation of Annealing Reactions and Analysis of Translation Products.

Translation of both Z4ENT and A20 ENT transcripts was performed using wheat germ lysate and rabbit reticulocyte lysate systems (Promega). Although both systems produced detectable protein when the products of translation were analyzed by SDS-PAGE and autoradiography, the rabbit reticulocyte system translated both the Z4ENT and A20ENT transcripts more efficiently than the wheat germ system.

Translation of the annealed samples was performed *in vitro*, using a nuclease-treated rabbit reticulocyte lysate system (Promega), and ³⁵S methionine was used to label the translation products (Amersham). The reactions were performed essentially according to the Promega protocol with modifications as described below.

To analyze translation products, reactions were run on SDS-PAGE, using a 4% stacking gel and 15% separation gel, with 0.75 or 1.5 mm spacers. Gels were run on a Hoefer apparatus, at 35 mA with constant current, for 3 to 3.5 hours. Samples were prepared for electrophoresis by adding 10 µl of each reaction to 40 µl of 1x sample buffer, then boiling for 7 minutes before spinning for 30 seconds in a microfuge. After removal of the stacking gel, gels were incubated for 30 minutes with shaking in a solution of 1 M sodium salicylate to enhance detection of the radioisotope. Gels were then rinsed briefly in water and dried on a slab drying under vacuum, at 65°C for 2 hours. The dried gels were exposed to film overnight,

using intensifying screens (Lightning Plus, Dupont Cronex) at -70°C. After developing, the gels were scanned using an LKB 2202 Ultrosan laser densitometer, and the data was compiled and analyzed using the Maxima software for chromatographic analysis (Waters Co.).

5 The results of *in vitro* translation of linearized plasmids containing the complete copies of the Z4 and A20 genes in the sense orientation show that the *in vitro* translation systems could be used to monitor the effects of antisense constructs on translation of the zein genes. Both translation systems produced proteins of the expected 19 kD weight species corresponding to the mature A20
10 gene product. Interestingly, however, while the rabbit reticulocyte system translated the Z4ENT transcript into the 22 kD preprotein, the wheat lysate system processed the Z4 preprotein, removing the signal peptide to produce the mature zein, resulting in a protein of approximately the same size as the 19 kD. In both systems, translation of the A20ENT transcript was at least 2-5X more efficient than
15 translation of the Z4ENT transcript, probably due to the lack of a signal peptide in the A20ENT protein or differences in accessibility of the start codon between the two transcripts, since the A20ENT transcript did not contain 5' noncoding sequence.

 Capping of the Z4ENT and A20ENT transcripts was performed as a possible means of increasing translation efficiency, using both cotranscriptional and
20 posttranscriptional procedures. No increase in translation efficiency was observed with either method.

 Hybrid arrest translations were performed using Z4ENT sense transcripts and Z4ENT antisense transcripts to establish annealing and translation conditions. A titration experiment was performed to determine the ratio of
25 antisense:sense transcripts needed to completely abolish Z4 synthesis. Amounts of antisense transcript were added to 1, 2, and 5-fold excess over the amount of sense transcript and allowed to anneal under controlled conditions. Results of this experiment are shown in Table III. Subsequent experiments, using a 4:1 ratio of

antisense:sense in the annealing reactions, were found to eliminate Z4 synthesis also, and so this ratio was used for later experiments.

TABLE III

**Effect of Increasing the Ratio of
Antisense to Sense Transcript on Z4 Synthesis**

			% Reduction in Z4 Synthesis	
			Range	Mean
5	Transcripts	Ratio		
	Z4ENTs	na	na	na
	Z4ENTas/Z4ENTs	1:1	55 - 63	59
	Z4ENTas/Z4ENTs	2:1	84 - 85	85
	Z4ENTas/Z4ENTs	5:1	100	100

Experiments were also done to determine whether the radiation dose/film exposure plot was sufficiently linear to allow quantitation of protein using laser densitometer readings of the film. To test this, the amount of extract loaded per lane was varied over a 25-fold range. Results indicated that the dose/response plot was acceptable over a 10-fold range only. Densitometry of the autoradiograms indicated that an overnight exposure of gels to film produced a meaningful dose-response curve, but that longer exposures did not.

Having established a basic protocol using the complete, perfectly complementary Z4ENT sense and antisense transcripts, a series of experiments was initiated to compare these results with the effect of antisense transcripts made from constructs containing only a portion of the Z4 transcriptional unit, as well as with antisense transcripts made from constructs containing all or portions of the A20 transcriptional unit. Data was compiled from several hybrid arrest of translation

experiments, all performed using a 4:1 molar ratio of antisense:sense transcript, and all incorporating the Z4ENT sense transcript with no antisense transcript added as a negative control (representing 100% synthesis of Z4, or 0% reduction in Z4 synthesis), and the Z4ENT transcript with the Z4ENT antisense transcript added as a positive control (representing 100% reduction in Z4 synthesis). A lambda transcript and a polylinker transcript were used as controls. The results are shown in Table IV.

The results are shown in Table IV.

TABLE IV

Hybrid Arrested Translation

Compiled Densitometer Data for
Reduction in Z4 Protein Synthesis

	<u>Antisense Transcript</u>	<u>Mean Reduction (%)</u>	<u>Number of Experiments Performed</u>
	Z4ENT	100	5
	Z45'	80	3
	Z4R3'	75	3
	A20ENT	81	3
	A20R	59	2
	Z10E	42	2
	lambda transcript	32	1
	polylinker transcript	0	2

General conclusions about the results can be drawn by summing the entire data set to generate a single rough consensus for efficiency of the antisense transcripts in effecting shutdown of Z4 synthesis, which are as follows:

Z4ENT>Z45' >A20ENT>Z4R3' >A20R>>Z10ENT>lambda> polylinker

This data indicates that the entire complementary transcript, as expected, is most efficient at reducing translation, and that antisense transcripts annealing to the translation initiation sequence are generally more efficient than transcripts annealing to the downstream coding region.

EXAMPLE 4

Production of Reagent Antibodies for Analysis of Maize Transformants

In order to screen for effects of antisense gene expression on zein expression levels in transformed cell lines and plants, polyclonal antibodies reactive with both the targeted α -zeins and with total zeins were produced. Antigens were extracted and purified as described below before inoculation into rabbits and subsequent antiserum characterization.

A. Antigen purification

Total zeins were obtained by extraction of the maize inbred line BSSS53. In this procedure, 4 grams of dry kernels were ground to a fine powder in a Braun coffee mill, defatted by incubation with 15 ml/g of acetone, with stirring, for 90 minutes at room temperature. The defatted meal was then filtered through a Buchner funnel and allowed to dry. Two extractions with 10 ml/g of 0.5 M NaCl were then performed; the mixture was stirred at room temperature for 30 minutes before filtering as above. Finally, two extractions were performed on the meal with 10 ml/g each of 70% ethanol % BME, for 60 minutes each, at room temperature with stirring. The ethanol extracts, totaling 80 ml, were pooled and filtered through a 0.45 micron filter before reducing the volume in a rotary evaporator (Rotovapor R110, Buchi Corp.). Evaporation was performed at 65°C, and after approximately 45 minutes the volume was reduced to 20 ml of solution, which had a cloudy appearance. This solution was diluted to 40 ml with sterile deionized water before

freezing and lyophilization. A dry weight of 329 mg was obtained, and a 1 mg sample was weighed out, resuspended in 1 ml of 70% ethanol, and protein content was quantitated by the Peterson assay (Peterson, 1979). The zeins were found to comprise 45% of the sample dry weight, and so approximately 140 mg of zein was obtained. Samples containing a range of 2.5 to 25 μ g of protein were analyzed for purity and presence of the expected zein profile by SDS-PAGE and silver or Coomassie blue staining of the gels (Sambrook et al., 1989). The preparation displayed the expected protein profile, with the 27 kD, 19/22 kD, 16 kD, 15 kD, and 10 kD zeins all present in the expected proportions. This preparation was, therefore, used as the antigen in raising of polyclonal sera against total zeins.

The α -zeins (19/22 kD zeins) were extracted from the maize inbred line A654 seed as follows: 6 grams of dry kernels were ground and processed as above for total zeins, from which approximately 500 mg of lyophilized sample was obtained. After determining protein content, the zeins were found to comprise 80% of the dry weight of the sample. To purify the α -zeins from the rest of the zeins, the sample was subjected to preparative SDS-PAGE: 10 mg of sample was weighed out, resuspended in 500 μ l of sample buffer/5% BME, then boiled for 10 minutes to eliminate aggregates before spinning for 30 seconds in the microfuge. Aliquots of 55 μ l/lane were run on a 3 mm thick gel, with a 4% stacker and a 15% separation gel. Extra long plates (25 cm long by 14 cm wide) were used to improve resolution. After running at 50 mA constant current for 3 hours, the gel was run at 15 mA overnight. Proteins were visualized by staining with cold 0.25 M TCA for approximately 10 minutes. Bands in the 19/22 kD range were then excised and washed in SDS gel running buffer until the gel pieces appeared clear. This buffer was saved, gel pieces were transferred to 2000 m.w. cutoff dialysis tubing. An additional 25 mg of starting material was processed in this fashion also, and all gel slices were pooled before dialysis. The dialysis tubing was sealed with clips, and placed in a Biorad mini-sub gel apparatus with the clips oriented perpendicularly with respect to the direction of electrophoresis. SDS running buffer was added to

the level of the tubing, and elution was performed at 10 mA overnight. The electrodes were reversed briefly, then the buffer inside the dialysis bag was pooled with the reserved buffer from the initial gel slice washes and dialyzed against 1 liter of deionized water, changing the water five times over several hours. The dialysate was lyophilized, the protein was quantitated, and then examined for purity by SDS-PAGE and silver staining. No contaminating protein species were visible, and so the purified antigen was used to inoculate rabbits for polyclonal antibody production. The total amount of purified α -zein obtained from this procedure was 10.9 mg, resulting in a yield of 31% for the procedure.

B. Antigen preparation and injection

A total of six New Zealand white rabbits were used for antibody production. Three were injected with purified α -zeins, and the remaining three were injected with purified total zeins as described below. Two of the six rabbits were treated using the traditional Freund's complete and incomplete adjuvant, and the remaining four were treated with a synthetic adjuvant, as described below.

Both α - and total zeins were weighed, resuspended, and heated to 65°C to completely solubilize the zeins; 0.5 mg of purified α -zein or 1.0 mg of total zein was resuspended in 60 μ l of 70% ethanol for each rabbit to be injected. Rabbits 1-3 received total zein as the antigen, and rabbits 4-6 received purified α -zein antigen. For rabbits 1 and 4 (designated 1F and 4F hereinafter), 440 μ l of PBS/Tween (phosphate buffered saline/2% Tween 80, Sigma) was added to the zein solution, then 500 μ l of Freund's complete adjuvant (Sigma) was added and the tubes were vortexed vigorously. The remaining four samples were made up as follows: to the 60 μ l of purified or total zein solution, 50 μ l of AVRIDINE (a synthetic adjuvant from Kodak) made up in 100% ethanol to 140 mg/ml, 760 μ l of Intralipid 10% fat emulsion (Travenol), and 300 μ l of PBS/Tween were added. After vortexing, the samples were sonicated in a cup sonicator for 2-30 second bursts (Ultrasonics, Inc.) to ensure complete emulsion before injection.

Samples were administered in 100 μ l aliquots injected at multiple sites across the back of the animals. Boosts were administered every three weeks, following the procedure above for formulating injection mixes except that Freund's incomplete adjuvant replaced the complete adjuvant for rabbits 1F and 4F. A total of three boosts were administered, in addition to the primary injection. Small volume (less than 5 ml) bleeds were performed to obtain sera for monitoring antibody titer and specificity during the process. Specificity and titer of the antisera were analyzed by running total zeins on SDS-PAGE/Western blots, as described below. Once titers were found to be sufficient (reactive at a 1:1000 sera dilution), several consecutive large (50 ml) bleeds were performed.

C. Analysis of Antisera

To determine antisera immunoreactivity and titer, total zein was assayed by SDS-PAGE/Western, with antisera dilutions from 1:50 to 1:1000 tested. The basic procedure was as follows: 500 ng of total zein/lane was dissolved in 10 μ l of sample buffer/2% BME, boiled 7.5 minutes, then loaded on a 15% minigel (Mini Protean II, BioRad) with molecular weight markers (BRL) in alternate lanes, and run at 200 V for 45 minutes. The stacking gel was removed, and the gel was equilibrated in transfer buffer (0.025 M TrisCl, 0.194 M glycine, 20% methanol) for 10 minutes before being overlaid with a prepared membrane (Millipore Immobilon-P). Preparation of the membrane was performed by rinsing with methanol, according to the manufacturer's recommendations, before equilibrating in transfer buffer. Proteins were transferred at 27 V for 40 minutes in a Genie electroblotter (idea Scientific). After transfer, membranes were rinsed and blocked in 3% BSA/PBS for one hour at 37°C on a shaker platform. Membranes were divided into strips by cutting at lanes containing molecular weight markers, and incubated with 10 ml of test antisera of the appropriate dilution overnight at 4°C, as well as with control polyclonal antisera directed against total zein. After removal of the primary antisera, membrane strips were washed in 1x PBS, for 5 x 10 minute

5 incubated in 4-chloro-naphthol substrate solution (KPL) until color development was complete, approximately 2-5 minutes. Reactions were stopped by rinsing the strips in deionized water.

immunolabelled only the 19/22 kD zeins, and not the other zeins (indicating that the quality of the gel purified antigen was at least as good as predicted by silver staining of SDS-PAGE, since antibody production would actually be a more sensitive measurement of contamination with other protein species). In addition, the sera from rabbits 4F, 5, and 6 exhibited reactivity with all of the zeins in approximate proportion to the relative amounts of protein present in the profile, showing slight to moderate labeling of the 27 kD zein, very strong labeling of the abundant 19/22 kD zeins, moderate labeling of the 16 and 14 kD zeins, and slight labeling of the less abundant 10 kD zein.

immunolabelling of blots with dilutions ranging from 1:50 to 1:1000 (for later bleeds). Although the lower dilutions of antisera immunolabelled the same zeins as the corresponding sera at higher dilutions, background staining of the membrane increased at sera dilutions of less than 1:500. Since the expected immunoreactivity profiles (as discussed above) were obtained at the 1:1000 dilution, this dilution was used for further analyses. Testing of the sera at dilutions of 1:2000 and higher might be indicated if sera conservation is desired, since dilutions of more than 1:1000 were not tested in these experiments.

The total amounts of sera obtained from the animals were as follows:
40 ml each of sera from rabbits 1, 4, and 6, and 80 ml each of sera from rabbits 2, 3,

and 5. The latter rabbits were chosen for further bleeds because the immunoreactivity profiles appeared to be slightly more specific to the α -zeins in the case of sera from rabbits 2 and 3 than was serum from rabbit 1F (which may have shown a very slight reactivity with the 10 kD zein), and slightly more reactive with the 10 kD zein in the case of sera from rabbits 5 and 6 than was sera from rabbit 4F.

EXAMPLE 5

Transformation of Maize with Z10 Promoter-Antisense Constructs

Embryogenic maize type II cultures were initiated from immature embryos isolated from developing seed derived from a cross of the genotypes B73 and A188 as described in PCT publication WO 95/06128 and U.S. application Serial No. 08/112,245. Type II cultures were microprojectile bombarded with a combination of plasmid vectors pDPG340 (Z10 promoter-Z4 antisense DNA sequence, described above) or pDPG380 (Z10 promoter-A20 antisense DNA sequence, described above) and pDPG363 comprising a plant expression cassette containing the Cauliflower Mosaic Virus 35S promoter operably linked in 5' to 3' order to intron 1 from the maize alcohol dehydrogenase I gene, the *bar* gene isolated from *Streptomyces hygroscopicus*, and the 3' terminator and polyadenylation sequences from the nopaline synthase gene of *Agrobacterium tumefaciens*. Transformed cell lines were selected for resistance to the herbicide bialaphos conferred by expression of the *bar* gene as described in U.S. Patent No. 5,489,520, U.S. Patent No. 5,550,318, and PCT publication WO 95/006128. Transformation of maize is further described in U.S. Patent No. 5,538, 877, U.S. Patent No. 5,538,880, and PCT publication WO 95/06128, the disclosures of which are incorporated by reference herein. The identification of transformed cell lines can be accomplished by employing selectable or screenable markers, as described hereinabove.

The presence of the antisense DNA sequence in transformants was verified by polymerase chain reaction (PCR). The sequence of the 5' PCR primer

was TCTAGGAAGCAAGGACACCACC (SEQ ID NO:5). The sequence of the 3' PCR primer was GCAAGACCGGCAACAGGATTCA (SEQ ID NO:6). The PCR reaction produced a DNA fragment of size about 1.0 kilobases in transformants containing pDPG380 and a DNA fragment of about size 1.1 kilobases in pDPG340 transformants.

Transformed callus lines containing antisense DNA sequences operably linked to a Z10 promoter were used to generate plants and seeds. Generally plants are regenerated as follows. Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, were cultured in media that supports regeneration of plants. In an exemplary embodiment, the inventors modified MS and N6 media (see Table 1 of U.S. application Serial No.08/594,861, the disclosure of which is incorporated by reference herein) by including further substances such as growth regulators. A preferred growth regulator for such purposes is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or picloram. Media improvement in these and like ways was found to facilitate the growth of cells at specific developmental stages. Tissue was preferably maintained on a basic media with growth regulators until sufficient tissue was available to begin plant regeneration efforts, or following repeated rounds of manual selection, until the morphology of the tissue is suitable for regeneration, at least two weeks, then transferred to media conducive to maturation of embryoids. Cultures were transferred every two weeks on this medium. Shoot development will signal the time to transfer to medium lacking growth regulators.

The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, were then allowed to mature into plants. Developing plantlets were transferred to soilless plant growth mix, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO₂, and 25-250 microeinsteins m⁻² s⁻¹ of light. Plants were preferably matured either in a growth chamber or greenhouse. Plants were

regenerated from about 6 weeks to 10 months after a transformant is identified, depending on the initial tissue. During regeneration, cells were grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels were petri dishes and Plant Con[®]s. Regenerating plants were preferably grown at about 19° to 5 28°C. After the regenerating plants reached the stage of shoot and root development, they were transferred to a greenhouse for further growth and testing.

By providing fertile, transgenic offspring, one can subsequently, through a series of breeding manipulations, move a selected gene from one corn line into an entirely different corn line without the need for further recombinant 10 manipulation. Movement of genes between corn lines is a basic tenet of the corn breeding industry, involving simply back crossing the corn line having the desired gene (trait). Introduced transgenes are valuable in that they behave genetically as any other corn gene and can be manipulated by breeding techniques in a manner identical to any other corn gene. Transformants containing Z10 promoter antisense 15 constructs (pDPG340 and/or pDPG380) were crossed to various maize inbred lines, including elite inbred lines designated AW, CN, CV, and DD.

Zein proteins were extracted from mature kernels from a maize plant transformed with plasmids pDPG340 and pDPG380 and crossed to inbreds AW or CN, according to Tsai (1980), as follows. Fifty milligrams of ground kernel was 20 suspended in 0.5 ml 70% ethanol, 1% β -mercaptoethanol and extracted at room temperature for 30 minutes to overnight. The sample was vortexed, centrifuged at 12,000 rpm for 5 minutes. Fifty microliters of the supernatant containing zein proteins was removed and dried. Zein proteins were resuspended in 50 μ l SDS polyacrylamide gel loading buffer containing 1% β -mercaptoethanol. Protein was 25 separated on SDS polyacrylamide gels and stained with Coomassie blue. No qualitative differences were observed in the amounts of 19 kD and 22 kD α -zein proteins (Figure 5). Furthermore, overall protein expression in the kernel appears to be the similar in antisense transformants and untransformed maize lines.

Analysis of the amino acid composition of Z10-antisense DNA transformants was undertaken. Amino acids were extracted from mature kernels as described in Jarrett et al., 1986; Jones et al., 1983; AACC, 1995). Results are summarized in Table V. Data was analyzed by t-tests and differences noted between transformed and untransformed kernels that were significant at the $p < 0.05$ level of significance. Transformed and untransformed kernels are from the same ear. The level of leucine only was statistically significantly decreased in transformant DD021. The level of lysine was statistically significantly increased and the level of leucine statistically significantly decreased in transformants DD015 and DD018.

These results are expected if expression of α -zeins is depressed in antisense transformants and expression of other proteins in the endosperm are increased. α -Zein proteins are rich in leucine residues and therefore one would expect that in the presence of reduced expression of α -zein proteins, the level of leucine would decrease in the kernel. Similarly, non-zein proteins contain more lysine than zein proteins and therefore increased expression of non-zein proteins results in increased lysine levels in the kernel. Therefore, the amino acid composition data relating to Z10-antisense transformants is consistent with a slight reduction in α -zein expression and increased expression of non-zein proteins, resulting in decreased levels of leucine and increased levels of lysine in the seed. Similar increases in lysine and decreases in leucine levels are observed in the maize *opaque-2* mutants in which zein synthesis is depressed and synthesis of non-zein proteins is increased. *Opaque-2* mutants, however, exhibit other phenotypic differences from wild type maize (Di Fonzo et al., 1988; Bass et al., 1992).

TABLE V

Transformant		Lysine ^{a,b}		Leucine ^{a,b}	
		Transformed	Untransformed	Transformed	Untransformed
5	DD015	1.96*	1.75	11.68*	13.97
		2.13	1.90	11.69*	14.50
	DD021	2.40	2.09	15.90*	17.90
		2.13	2.03	16.97	17.75
10	DD038	1.96	2.00	12.66	13.89
		1.82	1.96	15.87	15.73
	DD018	2.74*	2.43	17.57	19.13
		2.30*	2.15	13.19*	15.52

^a All amino acid concentrations are expressed as milligrams amino acid per gram of seed.

^b Asterisk denotes that amino acid concentration is statistically significantly different from the amino acid concentration in an untransformed kernel. T-tests were performed to compare amino acid concentrations in isogenic transformed and untransformed kernels. Statistically significant differences are those for which $p < 0.05$.

EXAMPLE 6

Transformation of Maize with Z27 Promoter-Antisense Expression Cassettes

Maize plants of the genotype A188 x B73 were crossed to Hi-II maize plants (Armstrong et al., 1991). Immature embryos (1.2 - 2.0 mm in length) were excised from surface-sterilized, greenhouse-grown ears of Hi-II 11-12 days post-pollination. The Hi-II genotype was developed from an A188 x B73 cross for high frequency development of type II callus from immature embryos (Armstrong et al., 1991). Approximately 30 embryos per petri dish were plated axis side down on a modified N6 medium containing 1 mg/l 2,4-D, 100 mg/l casein hydrolysate, 6 mM L-proline, 0.5 g/l 2-(N-morpholino)ethanesulfonic acid (MES), 0.75 g/l MgCl₂, and 2%

sucrose solidified with 2 g/l Gelgro, pH 5.8 (#735 medium) Embryos were cultured in the dark for two to four days at 24°C.

Approximately four hours prior to bombardment, embryos were transferred to the above culture medium with the sucrose concentration increased from 3% to 12%. When embryos were transferred to the high osmoticum medium they were arranged in concentric circles on the plate, starting 2 cm from the center of the dish, positioned such that their coleorhizal end was orientated toward the center of the dish. Usually two concentric circles were formed with 25-35 embryos per plate.

Gold particles were prepared containing 10 µg pDPG165 (described in U.S. Patent No. 5,489,520), and 10 µg of pDPG530.

The plates containing embryos were placed on the third shelf from the bottom, 5 cm below the stopping screen in the bombardment chamber. The 1100 psi rupture discs were used. Each plate of embryos was bombarded once. Embryos were allowed to recover overnight on high osmotic strength medium prior to initiation of selection.

Embryos were allowed to recover on high osmoticum medium (735, 12% sucrose) overnight (16 - 24 hours) and were then transferred to selection medium containing 1 mg/l bialaphos (#739, 735 plus 1 mg/l bialaphos or #750, 735 plus 0.2M mannitol and 1 mg/l bialaphos). Embryos were maintained in the dark at 24C. After three to four week on the initial selection plates about 90% of the embryos had formed Type II callus and were transferred to selective medium containing 3 mg/l bialaphos (#758). Bialaphos resistant tissue was subcultured about every two weeks onto fresh selection medium (#758). Transformants were confirmed using PCR analysis to detect presence of plasmid pDPG530. PCR primers used to confirm presence of the Z27-antisense expression cassette in transformed tissue were as follows: 5'GCA CTT CTC CAT CAC CAC CAC 3' (SEQ ID NO:6) and 5'TAT CCC CTT TCC AAC TTT CAG 3' (SEQ ID NO:7).

PCR amplification of pDPG530 and pDPG531 transformants produced a DNA product of about 500 base pairs.

Transformants were regenerated as generally described in PCT publication WO 95/06128. Transformed embryogenic callus was transferred to regeneration culture medium (MS culture medium (Murashige and Skoog, 1962), containing 0.91 mg/L L-asparagine, 1.4 g/L L-proline, 20 g/L D-sorbitol, 0.04 mg/L naphthalene acetic acid (NAA) and 3 mg/L 6-benzylaminopurine). Cells were grown for about four weeks on this culture medium with a transfer to fresh medium at about 2 weeks. Transformants were subsequently transferred to MS0 culture medium (MS medium with no phytohormones added). Regenerated plants were transferred to soil as described previously in this application. Plants were crossed to maize inbred lines designated AW, CV, and DJ. Seed containing the Z27-antisense expression cassette were opaque in phenotype similar to kernels of *opaque-2* mutant kernels. Furthermore, seed resulting from crosses of hemizygous Z-27-antisense transformants to untransformed inbreds resulted in seed segregating for the opaque phenotype in correlation with the presence of the Z-27 antisense expression cassette DNA sequence.

Zein proteins were extracted from mature kernels from maize plants transformed with plasmids pDPG530 and crossed to inbreds AW or CV as follows. Fifty milligrams of ground kernel was suspended in 0.5 ml 70% ethanol, 1% β -mercaptoethanol and extracted at room temperature for 30 minutes to overnight. The sample was vortexed, centrifuged at 12,000 rpm for 5 minutes. Fifty microliters of the supernatant containing zein proteins was removed and dried. Zein proteins are resuspended in 50 μ l SDS polyacrylamide gel loading buffer containing 1% β -mercaptoethanol. Protein was separated on SDS polyacrylamide gels and stained with Coomassie blue. Reduced amounts of 19 kD and 22 kD α -zeins were observed in five analyzed transformants. A Coomassie blue stained polyacrylamide gel of pDPG530 transformants and isogenic controls is shown in Figure 6. In one transformant, designated KP014, expression of the 27 kD zein protein, a γ type zein

protein was also depressed, suggesting that expression of an antisense DNA sequence in a maize may reduce expression of a related family of genes, i.e., the α -zeins, but also a member of a related family of proteins, i.e., 27 kD zein. A similar reduction in 27 kD was also observed for sense DNA sequences (see Figure 10). Isogenic controls were segregating kernels derived from plants lacking pDPG530 DNA sequences, recovered from crosses of pDPG530 transformed plants to untransformed inbreds. Furthermore, overall protein expression in the kernel appears to be the greater in antisense transformants than in untransformed maize lines as evidenced by overall protein staining by Coomassie blue on polyacrylamide gels (Figure 7). Reduction of α -zein synthesis is observed in *opaque-2* mutants, but the reduction is much less than in Z4 antisense expressing maize transformants.

It is contemplated that antisense repression of zein protein synthesis in the seed is a result of reduction in the amount of zein RNA present in the cell and consequently less synthesis of zein proteins. Northern blot analysis was completed to determine the level of steady state zein RNA synthesis in pDPG580 transformants. Procedures for Northern blot analysis are described in Sambrook et al. (1989). RNA isolated from maize kernels 21 days after pollination was separated by agarose gel electrophoresis and blotted to a Nitrobind membrane. The blot was probed with the Z4 coding sequence. A Northern blot analysis of the KP015 transformant is shown in Figure 8. Darker signals on the autoradiograph, e.g., lanes 3, 9, and 14 (upper panel) and lanes 3, 5, 11, and 12 (lower panel), correspond to untransformed seeds which showed normal level of zein synthesis. Other lanes (lighter signals) correspond to kernels that showed reduced levels of zein synthesis and the opaque phenotype in seeds containing the expression cassette.

Analysis of the amino acid composition of Z27-antisense DNA transformants was undertaken. Amino acids were extracted from mature kernels derived from three independent transformed lines as follows. Fifty milligrams of ground corn meal was hydrolyzed in 1 ml 6N HCl under argon gas for 24 hours at 110°C. Samples were diluted to 50 ml and filtered through a 0.45 micron filter.

5
10
15

TABLE VI

Transformant		Lysine ^{a,b}		Leucine ^{a,b}	
		Transformed	Untransformed	Transformed	Untransformed
5	KP014	2.60*	2.08	13.94	16.85
		2.85*	2.22	15.03*	17.08
		3.09*	2.40	15.66	18.14
		2.94*	2.45	15.27*	19.14
		2.60	2.56	10.08	14.95
10		2.45*	2.08	9.21	10.58
15	KP015	1.90*	1.02	3.85*	7.80
		1.92*	1.02	3.86*	7.98
		1.48*	0.94	4.44*	5.87
		1.43*	1.01	4.32*	6.26
20	KP016	2.10*	1.52	8.58*	11.90
		2.17*	1.54	8.95*	11.65
		2.66*	2.03	14.16*	20.37
		2.76*	1.81	14.68*	18.66
		4.65*	2.14	11.01*	21.32
		4.51*	2.31	11.26*	23.28
		3.91*	2.22	12.96*	23.99
25		3.98*	2.36	13.29*	24.06
		2.47*	1.76	9.60*	16.55
		2.48*	1.70	9.70*	14.83

* Denotes differences from untransformed kernels that are statistically significant at the $p < 0.05$ level of confidence.

Endosperm cells in the maize kernel are comprised primarily of large starch granules and protein sequestered in protein bodies (Lopes and Larkins, 1993). Zein proteins are essential for maintaining structure of the protein bodies (Lending and Larkins, 1989). A reduction in the number of protein bodies present in endosperm cells derived from a Z27 promoter-antisense transformant was observed by light microscopy (Figure 9). This observation is further evidence that α -zein synthesis was reduced in the Z27 promoter-antisense DNA transformants.

EXAMPLE 7

Transformation of Maize with Z27 Promoter-Sense Expression Cassettes

In higher plants the phenomenon of co-suppression of gene expression has been described (Napoli et al., 1990). Co-suppression refers to the suppression of endogenous gene expression by expression of a transgenic sense DNA expression cassette. It was contemplated that a sense zein expression cassettes in maize may result in suppression of endogenous zein expression in a manner similar to that described in Example 6 following expression of an antisense expression cassette.

Plasmid vector pDPG531 comprises a Z27 promoter-Z4 sense coding sequence-nopaline synthase 3' region expression cassette. pDPG531 differs from pDPG530 in that the Z4 coding sequence is operably linked to the Z27 in the opposite orientation, i.e., pDPG531 is capable of being transcribed and translated into the 22 kD zein protein. Plasmid pDPG531 and pDPG165 were introduced into maize cells as described in Example 6. Transformants were selected and regenerated as described in Example 6. Plants were regenerated from three Z27-Z4 sense expression cassettes and crossed to inbreds designated AW, CV, and CN.

The amount of α -zein proteins present in untransformed and Z27-Z4 sense transformants was compared on Coomassie blue stained polyacrylamide gels as described previously in reference to analysis of antisense transformants. Sample preparation and analysis were performed as described in Example 6. Figure 10 shows a Coomassie blue stained polyacrylamide gel. Each lane represents zein proteins extracted from a single seed of a segregating population of untransformed and sense expression cassette transformed seed. Lanes 1 through 8 represent seed derived from the transformant designated KQ012, and lanes 13 through 19 represent seed derived from a second transformant designated KQ020. Lanes 9 through 12 represent untransformed maize seed. Lanes 3, 4, 7, 8, 14, and 15 represent sense expression cassette transformed seed in which the α -zein levels are surprisingly

greatly reduced in a manner comparable to that observed in antisense transformants. In addition to the unexpected reduction in zein protein concentration in sense transformants, seed with reduced zein content also generally exhibit the opaque phenotype, and a reduction in Z27 zein levels.

5 In order to further determine whether the phenotype of Z27
promoter- Z4 sense transformants was similar to antisense transformants, lysine and
leucine concentrations were analyzed in seed derived from individual kernels.
Amino acids were analyzed as described in Example 6. In one transformant,
designated KQ018, lysine and leucine levels were statistically the same in isogenic
10 transformed and untransformed seed. However, in a transformant designated
KQ012, lysine levels were statistically increased in the transformant and leucine
levels were statistically significantly decreased in the transformant. It is therefore
apparent, that the Z27 promoter- Z4 sense transformants produce a seed
morphology, protein, and amino acid composition phenotype, similar to that
15 observed in antisense transformants.

EXAMPLE 8

Method to Increase Content of Methionine in Plants

A method for increasing the methionine content of seeds involves cotransforming maize tissue culture with a zein sense or antisense DNA sequence (either A20 or Z4) and a DNA sequence containing a gene encoding a 10 kD zein protein. It is known that the 10 kD zein proteins are rich in methionine. A decrease in expression of A20 and/or Z4 zein proteins combined with an increase in expression in the 10 kD zein proteins is likely to lead to about a 50% to 300% increase in total weight percent of methionine in the seed.

Antisense or sense DNA sequences containing a DNA sequence complementary or homologous to A20 and/or Z4 have been prepared as described in

A DNA sequence containing a gene encoding a 10 kD zein protein was prepared as described in U.S. Patent No. 5,508,468, which is hereby incorporated reference. Preferably, a Z10 DNA sequence contains a gene encoding a 10 kD zein protein including the 3' noncoding sequence combined with the promoter from a 27 kD zein protein. A plasmid with this DNA sequence has been prepared and is designated pZ27Z10 and is described in U.S. Patent No. 5,508,468.

The expression of the chimeric Z10 gene at the RNA level in Met1 seeds was demonstrated. Immature endosperms (21 DAP) were harvested from a segregating ear of the background Met1 x A654 BC2. Both DNA and RNA were prepared from individual endosperm samples. The DNA was analyzed by PCR for the presence/absence of the Z27Z10 gene. The RNA samples were analyzed by Northern blot, probing with an oligonucleotide spanning the junction between the Z27 promoter and the Z10 coding region. The results demonstrate that the gene is expressed in endosperm tissue of PCR+ seeds and not in that of PCR- seeds.

Seeds containing a DNA sequence containing the 10 kD zein protein combined with the 27 kD promoter were field tested. A total of 130 ears were genotyped by PCR (using DNA from pooled leaf samples of germinated seedlings) and analyzed for methionine content by amino acid analysis, and 10 kD zein levels by ELISA. There is a positive correlation between 10 kD zein levels and methionine content in several maize backgrounds tested. It is, therefore, contemplated that if α -zein synthesis is reduced by expression of sense or antisense zein constructs, expression of a transgenic 10 kD zein will increase the methionine content of a seed. The results indicate that if it is possible to elevate the expression

of the 10 kD zein at least about 5-10 fold, methionine contents in maize seed can be significantly raised (up to 2.5 to 3%). Additional transformants with the 10 kD zein functionally linked to the 27 kD zein promoter and/or the Z4 22 kD zein promoter and/or the 10 kD zein promoter which show elevated levels of 10 kD zein and
5 methionine in transformed seed have also been generated as described above and in U.S. Patent No. 5,508,468.

Maize tissue cultures are cotransformed with a sense or antisense DNA sequence and a 10 kD zein DNA sequence and a selectable marker gene. Transformed cell lines containing both DNA sequences are identified by PCR
10 analysis.

The transformed cell lines positive for PCR analysis for both an antisense and the 10 kD zein DNA sequences are used to regenerate transformed plants and seeds, as described in Example 6. Seeds are analyzed for expression of 10 kD zein and Z4 (22 kD) using Western blots. Total methionine content of the
15 seed is determined as described in Examples 5 and 6.

An increase in the 10 kD zein expression combined with a decrease in the A20 and/or Z4 zein protein results in a significant increase (up to about 50 to 300%) in the total methionine content of the seed.

20 **EXAMPLE 9**

Method to Increase Amino Acid Content of Particular Amino Acids in Seeds

The amino acid content of seeds is increased by expression of a gene encoding a synthetic polypeptide that comprises one or more amino acids for which altered levels are desired in the seed. Amino acid content is altered by expression of
25 a gene encoding a naturally occurring or synthetic polypeptide comprising one or more desired amino acids, in a seed in which expression of endogenous seed storage

proteins have been repressed by expression of a sense or antisense seed storage protein DNA sequence.

For example, a gene encoding the synthetic protein MB1 is introduced into a plant in which storage protein synthesis is repressed by expression of a sense or antisense DNA sequence. The MB1 coding sequence is introduced into a transgenic plant with reduced expression of storage protein, wherein said plant was previously transformed with a storage protein sense or antisense DNA sequence. Alternatively, the MB1 sequence is transformed into a plant simultaneously with a storage protein sense or antisense DNA sequence. In a preferred embodiment of the present invention, a storage protein antisense or sense expression cassette and an MB1 expression cassette are transformed into maize simultaneously or sequentially as described in Examples 5, 7, and 8.

A plasmid vector, designated pDPG780, containing an MB1 plant expression cassette was constructed. The MB1 protein coding sequence was obtained from Mary A. Hefford (Center for Food and Animal Research, Agriculture and Agri-Food Canada, Ottawa, ON, K1A 0C6, Canada) and the DNA sequence is disclosed in Beauregard et al., 1995. MB1 is a synthetic protein enriched in methionine, threonine, lysine and leucine and exhibits α -helical structure similar to a zein protein. Plasmid vector pDPG780 was constructed by operably linking an endoplasmic reticulum signal sequence (Pedersen et al., 1986) from the 15 kD zein protein encoding gene 5' to the MB1 coding sequence. The 15 kD zein-MB1 sequence was inserted in plasmid vector pZ27-nos between the Z27 promoter element and the nopaline synthase 3' region (nos). The expression cassette comprises in 5' to 3' orientation, the Z27 promoter, Z15 signal sequence, MB1 coding sequence, and nos 3' region. One of skill in the art could construct additional plasmid vectors containing a seed specific promoter operably linked to an endoplasmic reticulum signal sequence, protein encoding sequence, and 3' region, wherein said protein encoding sequence comprises a DNA sequence encoding a protein of desired amino acid composition.

The plasmid vector pDPG780 is introduced into maize in conjunction with a vector comprising a selectable marker gene, e.g., pDPG165 comprising the *bar* gene. The MB1 expression cassette is transformed into maize plants containing a sense or antisense zein transgene in which synthesis of α -zein proteins is repressed. Alternatively, the sense or antisense zein construct is transformed into maize simultaneously with the MB1 expression cassette.

Plants are regenerated as described in Examples 5, 6 and 7. Protein composition of seed is analyzed by polyacrylamide gel electrophoresis as described in Examples 5 and 6. Reduction in zein proteins is observed and expression of a protein of desired amino acid composition is observed. Amino acid composition of seed is determined as described in Examples 5, 6, and 7. Levels of desired amino acids are altered in accordance with the amino acid composition of the protein encoded by the transgene.

While the present invention has been described in connection with the preferred embodiment thereof, it will be understood many modifications will be readily apparent to those skilled in the art, and this application is intended to cover any adaptations or variations thereof. All patents, patent documents and publications described herein are hereby incorporated by reference.

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WHAT IS CLAIMED:

1. An expression cassette comprising a preselected DNA sequence encoding an RNA molecule operably linked to a promoter functional in a host cell,
5 wherein the RNA molecule is substantially complementary to all or a portion of a mRNA encoding a plant seed storage protein.
2. An expression cassette comprising a preselected DNA sequence encoding an RNA molecule operably linked to a promoter functional in a host cell,
10 wherein the RNA molecule is substantially identical to all or a portion of a mRNA encoding a plant seed storage protein.
3. The expression cassette of claim 1 or 2 in which the plant seed storage protein is a maize seed storage protein.
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4. The expression cassette of claim 3 wherein the maize seed storage protein is an α -zein protein.
5. The expression cassette of claim 1 or 2 which further comprises a selectable
20 marker gene.
6. The expression cassette of claim 1 or 2 further comprising plasmid DNA.
7. The expression cassette of claim 1 or 2 wherein the promoter is a promoter
25 functional during plant seed development.

8. The expression cassette of claim 1 wherein the promoter comprises the 10 kD zein promoter.
9. The expression cassette of claim 1 wherein the promoter comprises the 27 kD zein promoter.
10. The expression cassette of claim 1, 8 or 9 wherein the preselected DNA sequence encodes an RNA molecule that is substantially complementary to all or a portion of a mRNA for a 19 kD α -zein protein.
11. The expression cassette of claim 1, 8 or 9 wherein the preselected DNA sequence encodes an RNA molecule that is substantially complementary to all or a portion of a mRNA for a 22 kD α -zein protein.
12. The expression cassette of claim 2, 8 or 9 wherein the preselected DNA sequence encodes an RNA molecule that is substantially identical to all or a portion of a mRNA for a 19 kD α -zein protein.
13. The expression cassette of claim 2, 8 or 9 wherein the preselected DNA sequence encodes an RNA molecule that is substantially identical to all or a portion of a mRNA for a 22 kD α -zein protein.
14. The expression cassette of claim 2 or 9 wherein the preselected DNA sequence encodes MB1.

15. The expression cassette of claim 1 or 2 which further comprises a second preselected DNA sequence which encodes kernel hardness.
16. A method for increasing the weight percent of at least one amino acid which is essential to the diet of animals in plant seed, comprising:
- a) stably transforming plant cells with an expression cassette comprising a preselected DNA sequence encoding an RNA molecule operably linked to a promoter functional in the plant cell so as to yield a transformed plant cell, wherein the RNA molecule is substantially identical, or complementary, to all or a portion of a mRNA encoding a seed storage protein;
 - b) regenerating the transformed cells into a fertile transgenic plant which produces seeds, in which the preselected DNA sequence is expressed in the seeds in an amount sufficient to decrease the weight percent of the seed storage protein relative to the weight percent of the seed storage protein present in seeds of a corresponding nontransgenic plant; and
 - (c) recovering said transgenic seeds from said transgenic plant.
17. A method for increasing the weight percent of starch in a seed, which comprises:
- a) stably transforming plant cells with an expression cassette comprising a preselected DNA sequence encoding an RNA molecule operably linked to a promoter functional in the plant cell so as to yield transformed plant cells, wherein the RNA molecule is substantially identical, or complementary, to all or a portion of a mRNA encoding a seed storage protein;

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comprising a preselected DNA sequence encoding an RNA molecule operably linked to a promoter functional in a plant cell so as to yield transformed plant cells, wherein the RNA molecule is substantially identical, or complementary, to all or a portion of a messenger RNA for the plant seed storage protein;

(b) regenerating the transformed cells into a fertile transgenic plant which generates plant seeds, wherein the preselected DNA sequence is expressed in the seeds in an amount effective to substantially reduce expression of the plant seed storage protein; and

(c) recovering said seeds.

20. The method of claim 16, 17, 18 or 19 wherein the preselected DNA segment encodes an RNA molecule that is substantially identical to all or a portion of the mRNA encoding a seed storage protein.

21. The method of claim 16, 17, 18 or 19 wherein the preselected DNA segment encodes an RNA molecule that is substantially complementary to all or a portion of the mRNA encoding a seed storage protein.

22. The method of claim 20 wherein the preselected DNA segment encodes an RNA molecule that is substantially identical to all or a portion of the mRNA encoding an α -zein protein.

23. The method of claim 21 wherein the preselected DNA segment encodes an RNA molecule that is substantially complementary to all or a portion of the mRNA encoding an α -zein protein.

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32. The method of claim 21 wherein the preselected DNA sequence encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding a 19 kD α -zein protein.
- 5 33. The method of claim 21 wherein the preselected DNA sequence encodes an RNA molecule substantially complementary to all or a portion of a messenger RNA encoding a 22 kD α -zein protein.
- 10 34. The method of claim 20 wherein the preselected DNA sequence encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a 19 kD α -zein protein.
- 15 35. The method of claim 21 wherein the preselected DNA sequence encodes an RNA molecule substantially identical to all or a portion of a messenger RNA encoding a 22 kD α -zein protein.
36. The method of claim 16, 17, 18 or 19 which further comprises stably transforming the cells with a second preselected DNA sequence which encodes kernel hardness.
37. The method of claim 16, 17, 18 or 19 wherein the cell is transformed by a method selected from the group consisting of electroporation, microinjection, microprojectile bombardment, and liposomal encapsulation.

38. The method of claim 16, 17, 18 or 19 which further comprises stably transforming the cells with at least one selectable marker gene.
39. The method of claim 26 further comprising breeding the fertile transgenic plant to yield a progeny plant that has an increase in the weight percent of the at least one amino acid as a dominant trait while still maintaining functional agronomic characteristics relative to the corresponding untransformed plant.
40. The method of claim 17 or 18 further comprising breeding the fertile transgenic plant to yield a progeny plant that has an increase in the weight percent of starch as a dominant trait while still maintaining functional agronomic characteristics relative to the corresponding untransformed plant.
41. A method for inhibiting expression of a family of seed storage protein genes in a plant seed, which comprises:
- (a) stably transforming plant cells with a first preselected DNA sequence which encodes a RNA molecule operably linked to a promoter functional in a plant or seed so as to yield transformed plant cells, wherein the RNA molecule is substantially identical, or complementary, to all or a portion of a messenger RNA that encodes a polypeptide which is substantially homologous in seed storage proteins; and
 - (b) regenerating the transformed cells into a fertile transgenic plant which yields transgenic seeds, wherein the preselected DNA sequence is expressed in the seeds in an amount effective to substantially reduce expression of seed storage proteins in the transgenic seeds relative to the

expression of seed storage proteins in the corresponding nontransgenic seeds.

42. A method for increasing the weight percent of at least one amino acid essential to the diet of animals in a plant seed, which comprises:
- (a) stably transforming plant cells with a first preselected DNA sequence and a second preselected DNA sequence so as to yield transformed plant cells, wherein the first preselected DNA sequence encodes a RNA molecule substantially identical, or complementary, to all or a portion of a messenger RNA encoding a seed storage protein, wherein the second preselected DNA sequence encodes a polypeptide having at least one amino acid essential to the diet of animals, and wherein each preselected DNA sequence is operably linked to a promoter functional in a plant or seed; and
- (b) regenerating the transformed cell into a fertile transgenic plant which yields transgenic seeds, wherein the first preselected DNA sequence is expressed in an amount effective to substantially reduce the production of said seed storage protein in said transgenic seeds relative to the amount of said seed storage protein present in the corresponding nontransgenic seeds, and wherein the second preselected DNA sequence is expressed in an amount sufficient to increase the weight percent of the at least one essential amino acid in said transgenic seeds relative to the amount of said essential amino acid present in the corresponding nontransgenic seeds.
43. A method for increasing the production of a polypeptide in a seed, which comprises:
- (a) stably transforming plant cells with a first preselected DNA

sequence and a second preselected DNA sequence so as to yield a transformed cell, wherein the first preselected DNA sequence encodes an RNA molecule substantially identical, or complementary, to all or a portion of at least one messenger RNA encoding a seed storage protein, wherein the second preselected DNA molecule encodes a polypeptide, and wherein each preselected DNA sequence is operably linked to a promoter functional in said plant; and

(b) regenerating the transformed cells into a fertile transgenic plant which produces transgenic seeds, wherein the first preselected DNA sequence is expressed in the transgenic seeds in an amount effective to substantially reduce the production of the seed storage protein relative to the amount of said seed storage protein present in the corresponding nontransgenic seeds, and wherein the second preselected DNA sequence is expressed in said transgenic seeds as a protein in a weight percent which is substantially increased over the weight percent of that protein in the corresponding nontransgenic seeds.

44. The method of claim 41, 42 or 43 further comprising collecting the transgenic seeds.

45. The method of claim 41 wherein the polypeptide is substantially homologous in α -zein proteins.

46. The method of claim 42 or 43 wherein the first preselected DNA segment encodes an RNA molecule that is substantially identical to all or a portion of the mRNA encoding a seed storage protein.

47. The method of claim 42 or 43 wherein the first preselected DNA segment encodes an RNA molecule that is substantially complementary to all or a portion of the mRNA encoding a seed storage protein.
- 5 48. The method of claim 46 wherein the preselected DNA segment encodes an RNA molecule that is substantially identical to all or a portion of the mRNA encoding an α -zein protein.
- 10 49. The method of claim 47 wherein the preselected DNA segment encodes an RNA molecule that is substantially complementary to all or a portion of the mRNA encoding an α -zein protein.
- 15 50. The method of claim 41, 42 or 43 wherein the plant cell is a monocot cell.
51. The method of claim 50 wherein the cell is a maize cell.
- 20 52. The method of claim 42 in which the seeds of the transgenic plant have an increased weight percent of at least one essential amino acid.
53. The method of claim 52 wherein the essential amino acid is selected from the group consisting of methionine, threonine, lysine, tryptophan, isoleucine and mixtures thereof.
- 25 54. The method of claim 52 wherein the weight percent of the amino

acid is increased at least about 50% to 300%.

55. The method of claim 41, 42 or 43 wherein the preselected DNA sequence is operably linked to a promoter functional during plant seed development.

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56. The method of claim 41, 42 or 43 wherein the promoter comprises the 10 kD zein promoter.

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57. The method of claim 41, 42 or 43 wherein the promoter comprises the 27 kD zein promoter.

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58. The method of claim 49 wherein the preselected DNA sequence encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding a 19 kD α -zein protein.

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59. The method of claim 49 wherein the preselected DNA sequence encodes an RNA molecule substantially complementary to all or a portion of a messenger RNA encoding a 22 kD α -zein protein.

60. The method of claim 48 wherein the preselected DNA sequence encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a 19 kD α -zein protein.

61. The method of claim 48 wherein the preselected DNA sequence

encodes an RNA molecule substantially identical to all or a portion of a messenger RNA encoding a 22 kD α -zein protein.

- 5 62. The method of claim 42 or 43 wherein the second preselected DNA sequence encodes MB1.
63. The method of claim 42 or 43 wherein the second preselected DNA sequence encodes a 10 kD zein.
- 10 64. The method of claim 42 or 43 wherein the cells are stably transformed with a third preselected DNA sequence which encodes kernel hardness.
65. The method of claim 64 wherein the third preselected DNA sequence encodes a 27 kD zein protein.
- 15 66. The method of claim 41 wherein the cells are stably transformed with a second preselected DNA sequence which encodes kernel hardness.
- 20 67. The method of claim 66 wherein the second preselected DNA sequence encodes a 27 kD zein protein.
68. The method of claim 41, 42 or 43 wherein the cell is transformed by a method selected from the group consisting of electroporation, microinjection, microprojectile bombardment, and liposomal encapsulation.
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69. The method of claim 41, 42 or 43 wherein the cells are stably transformed with at least one selectable marker gene.
70. The method of claim 42 further comprising breeding the fertile transgenic plant to yield a progeny plant that has an increase in the weight percent of the at least one amino acid as a dominant trait while still maintaining functional agronomic characteristics relative to the corresponding untransformed plant.
71. A fertile transgenic *Zea mays* plant having an increased weight percent of at least one amino acid which is essential to the diet of an animal, the genome of which is stably augmented by a preselected DNA sequence encoding an RNA molecule which is substantially identical, or complementary, to a mRNA encoding a plant seed storage protein, wherein the preselected DNA sequence is expressed in the cells of the transgenic plant in an amount sufficient to decrease the amount of the seed storage protein relative to the amount of said seed storage protein in the cells of a plant which only differ from the cells of said transgenic plant in that said preselected DNA sequence is absent, and wherein said preselected DNA sequence is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.
72. A fertile transgenic *Zea mays* plant having an increased starch content, the genome of which is stably augmented by a preselected DNA sequence encoding an RNA molecule which is substantially identical, or complementary, to a mRNA encoding a plant seed storage protein, wherein the preselected DNA sequence is expressed in the cells of the transgenic

plant in an amount sufficient to decrease the amount of said seed storage protein and to increase the starch content relative to the amount of said seed storage protein and starch content in the cells of a plant which only differ from the cells of said transgenic plant in that said preselected DNA sequence is absent, and wherein said preselected DNA sequence is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.

73. A fertile transgenic *Zea mays* plant, the seeds of which have an increased starch extractability, the genome of said plant which is stably augmented by a preselected DNA sequence encoding an RNA molecule which is substantially identical, or complementary, to a mRNA encoding a plant seed storage protein, wherein the preselected DNA sequence is expressed in the seeds of the transgenic plant in an amount sufficient to decrease the amount of said seed storage protein and to increase the starch extractability of the seed relative to the amount of said seed storage protein and starch extractability in the seeds of a plant which only differ from the seeds of said transgenic plant in that said preselected DNA sequence is absent, and wherein said preselected DNA sequence is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.

74. A fertile transgenic *Zea mays* plant having a decreased amount of seed storage protein, the genome of which is stably augmented by a preselected DNA sequence encoding an RNA molecule which is substantially identical, or complementary, to a mRNA encoding a seed storage protein, wherein the RNA molecule is substantially identical, or complementary, to all or a portion of a mRNA molecule that encodes a peptide that is substantially homologous in seed storage proteins, wherein the preselected DNA sequence

is expressed in the cells of the transgenic plant in an amount sufficient to decrease the amount of seed storage proteins in the cells of a plant which only differ from the cells of said transgenic plant in that said preselected DNA sequence is absent, and wherein said preselected DNA sequence is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.

75. A fertile transgenic *Zea mays* plant having a decreased seed storage protein content, the genome of which is stably augmented by a preselected DNA sequence encoding an RNA molecule which is substantially identical, or complementary, to a mRNA encoding a seed storage protein, wherein the preselected DNA sequence is expressed in the cells of the transgenic plant in an amount sufficient to decrease the amount of said protein in the cells of a plant which only differ from the cells of said transgenic plant in that said preselected DNA sequence is absent, and wherein said preselected DNA sequence is transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.
76. A fertile transgenic *Zea mays* plant having an increased weight percent of at least one amino acid essential to the diet of an animal, the genome of which is stably augmented by a first preselected DNA sequence and a second preselected DNA sequence, wherein the first preselected DNA sequence encodes an RNA molecule which is substantially identical, or complementary, to a mRNA encoding a seed storage protein, wherein the second preselected DNA sequence encodes a polypeptide having at least one amino acid essential to the diet of an animal, wherein the first preselected DNA sequence is expressed in the cells of the transgenic plant in an amount sufficient to decrease the amount of said seed storage protein and the second

preselected DNA sequence is expressed in the cells of the transgenic plant in an amount sufficient to increase the weight percent of the at least one amino acid essential to the diet of an animal relative to the amount of said seed storage protein and weight percent of said essential amino acid in the cells of a plant which only differ from the cells of said transgenic plant in that said preselected DNA sequences are absent, and wherein said preselected DNA sequences are transmitted through a complete normal sexual cycle of the transgenic plant to the next generation.

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- 10 77. A fertile transgenic *Zea mays* plant having an increased amount of a preselected polypeptide, the genome of which is stably augmented by a first preselected DNA sequence and a second preselected DNA sequence, wherein the first preselected DNA sequence encodes an RNA molecule which is substantially identical, or complementary, to a mRNA encoding a
- 15 seed storage protein, wherein the second preselected DNA sequence encodes a preselected polypeptide, wherein the first preselected DNA sequence is expressed in the cells of the transgenic plant in an amount sufficient to decrease the amount of said seed storage protein and the second preselected DNA sequence is expressed in the cells of the transgenic plant in an amount
- 20 sufficient to increase the amount of said preselected polypeptide relative to the amount of said seed storage protein and said preselected polypeptide in the cells of a plant which only differ from the cells of said transgenic plant in that said preselected DNA sequences are absent, and wherein said preselected DNA sequences are transmitted through a complete normal
- 25 sexual cycle of the transgenic plant to the next generation.

78. A seed derived from the plant of claim 71, 72, 73, 74, 75, 76 or 77.

79. A progeny plant derived from the seed of claim 78.
80. The transgenic plant of claim 74 wherein the preselected DNA sequence encodes an RNA molecule which is substantially identical, or complementary, to all or a portion of an mRNA that encodes a peptide that is substantially homologous in α -zein proteins.
81. The transgenic plant of claim 71 or 75 in which the seeds of the transgenic plant have an increased weight percent of at least one essential amino acid.
82. The transgenic plant of claim 81 wherein the essential amino acid is selected from the group consisting of methionine, threonine, lysine, tryptophan, isoleucine and mixtures thereof.
83. The transgenic plant of claim 81 wherein the weight percent of the amino acid is increased at least about 50% to 300%.
84. The transgenic plant of claim 71, 72, 73, 74, or 75 wherein the promoter comprises the 10 kD zein promoter.
85. The transgenic plant of claim 76 or 77 wherein at least one promoter comprises the 10 kD zein promoter.
86. The transgenic plant of claim 71, 72, 73, 74 or 75 wherein the promoter comprises the 27 kD zein promoter.

87. The transgenic plant of claim 76 or 77 wherein at least one promoter comprises the 27 kD zein promoter.
88. The transgenic plant of claim 71, 72, 73, 74, 75, 76 or 77 wherein the preselected DNA sequence, which encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding a seed storage protein, encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding 19 kD α -zein protein.
89. The transgenic plant of claim 71, 72, 73, 74, 75, 76 or 77 wherein the preselected DNA sequence, which encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding a seed storage protein, encodes an RNA molecule substantially complementary to all or a portion of a mRNA encoding a 22 kD α -zein protein.
90. The transgenic plant of claim 71, 72, 73, 74, 75, 76 or 77 wherein the preselected DNA sequence, which encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a seed storage protein, encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a 19 kD α -zein protein.
91. The transgenic plant of claim 71, 72, 73, 74, 75, 76 or 77 wherein the preselected DNA sequence, which encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a seed storage protein, encodes an RNA molecule substantially identical to all or a portion of a mRNA encoding a 22 kD α -zein protein.

92. The transgenic plant of claim 76 or 77 wherein the second preselected DNA sequence encodes MB1.
93. The transgenic plant of claim 76 or 77 wherein the second preselected DNA sequence encodes a 10 kD zein.
94. The transgenic plant of claim 71, 72, 73, 74, 75, 76 or 77 further comprising stably transforming the cells with a gene which encodes kernel hardness.
95. The transgenic plant of claim 71, 72, 73, 74, 75, 76 or 77 wherein the cell is transformed by a method selected from the group consisting of electroporation, microinjection, microprojectile bombardment, and liposomal encapsulation.
100. The transgenic plant of claim 75, 76, 77, 78, 79, 80 or 81 further comprising stably transforming the cells with at least one selectable marker gene.

The invention provides genetically engineered, preselected DNA sequences and methods of using them to alter the nutritional content of plant seed. Methods of the invention are directed to increasing the weight percent of at least one amino acid essential to the diet of animals, or increasing the starch content, of a plant. One such method involves stably transforming a cell of a plant with an a preselected DNA sequence encoding an RNA molecule substantially identical or complementary to a messenger RNA (mRNA) encoding a plant seed storage protein, preferably a seed storage protein which is deficient in at least one amino acid essential to the diet of animals. An alternative method employs stably transforming cells with at least two preselected DNA sequences, one of which encodes an RNA molecule substantially identical or complementary to a messenger RNA (mRNA) encoding a plant seed storage protein, and the other preselected DNA molecule which encodes a preselected polypeptide. The transformed cells are used to generate fertile transgenic plants and seeds. Transgenic seeds are characterized by expression of the preselected DNA sequence which results in a substantial inhibition of production of a seed storage protein deficient in at least one amino acid essential to the diet of animals and/or an increase in the weight percent of an amino acid essential to the diet of animals.

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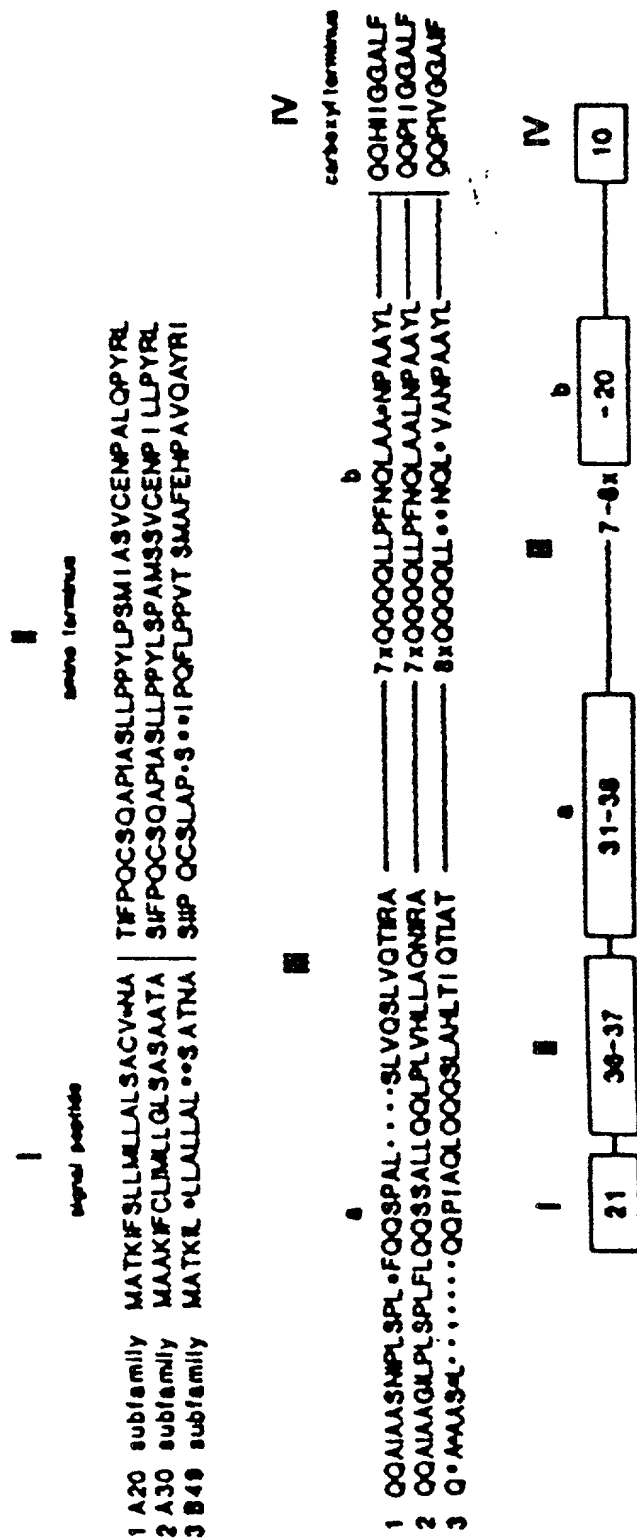


FIGURE 1

1990-1991		1991-1992		1992-1993		1993-1994		1994-1995		1995-1996		1996-1997		1997-1998		1998-1999		1999-2000		2000-2001		2001-2002		2002-2003		2003-2004		2004-2005		2005-2006		2006-2007		2007-2008		2008-2009		2009-2010		2010-2011		2011-2012		2012-2013		2013-2014		2014-2015		2015-2016		2016-2017		2017-2018		2018-2019		2019-2020		2020-2021		2021-2022		2022-2023		2023-2024		2024-2025		2025-2026		2026-2027		2027-2028		2028-2029		2029-2030		2030-2031		2031-2032		2032-2033		2033-2034		2034-2035		2035-2036		2036-2037		2037-2038		2038-2039		2039-2040		2040-2041		2041-2042		2042-2043		2043-2044		2044-2045		2045-2046		2046-2047		2047-2048		2048-2049		2049-2050		2050-2051		2051-2052		2052-2053		2053-2054		2054-2055		2055-2056		2056-2057		2057-2058		2058-2059		2059-2060		2060-2061		2061-2062		2062-2063		2063-2064		2064-2065		2065-2066		2066-2067		2067-2068		2068-2069		2069-2070		2070-2071		2071-2072		2072-2073		2073-2074		2074-2075		2075-2076		2076-2077		2077-2078		2078-2079		2079-2080		2080-2081		2081-2082		2082-2083		2083-2084		2084-2085		2085-2086		2086-2087		2087-2088		2088-2089		2089-2090		2090-2091		2091-2092		2092-2093		2093-2094		2094-2095		2095-2096		2096-2097		2097-2098		2098-2099		2099-2100		2100-2101		2101-2102		2102-2103		2103-2104		2104-2105		2105-2106		2106-2107		2107-2108		2108-2109		2109-2110		2110-2111		2111-2112		2112-2113		2113-2114		2114-2115		2115-2116		2116-2117		2117-2118		2118-2119		2119-2120		2120-2121		2121-2122		2122-2123		2123-2124		2124-2125		2125-2126		2126-2127		2127-2128		2128-2129		2129-2130		2130-2131		2131-2132		2132-2133		2133-2134		2134-2135		2135-2136		2136-2137		2137-2138		2138-2139		2139-2140		2140-2141		2141-2142		2142-2143		2143-2144		2144-2145		2145-2146		2146-2147		2147-2148		2148-2149		2149-2150		2150-2151		2151-2152		2152-2153		2153-2154		2154-2155		2155-2156		2156-2157		2157-2158		2158-2159		2159-2160		2160-2161		2161-2162		2162-2163		2163-2164		2164-2165		2165-2166		2166-2167		2167-2168		2168-2169		2169-2170		2170-2171		2171-2172		2172-2173		2173-2174		2174-2175		2175-2176		2176-2177		2177-2178		2178-2179		2179-2180		2180-2181		2181-2182		2182-2183		2183-2184		2184-2185		2185-2186		2186-2187		2187-2188		2188-2189		2189-2190		2190-2191		2191-2192		2192-2193		2193-2194		2194-2195		2195-2196		2196-2197		2197-2198		2198-2199		2199-2200		2200-2201		2201-2202		2202-2203		2203-2204		2204-2205		2205-2206		2206-2207		2207-2208		2208-2209		2209-2210		2210-2211		2211-2212		2212-2213		2213-2214		2214-2215		2215-2216		2216-2217	
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006230-0420960

- 002230 04820960
- A. PRIMER 1
5' CCCGGGTAGATAATATATCGCAC 3'
- PRIMER 2
5' CCCGGGCTGCCATTATTGGTACAATATTGTGCTTTCTATG 3'
- B. PRIMER 1
5' CCCGGGCAAACCTTGCATGCCTACTCTCAGC 3'
- PRIMER 2
5' CCCGGGTAGTAGTTGTTGTTGCATGCAAATAAGCAGC 3'
- C. PRIMER 1
5' CCCGGGTCTAGATTGCTTATGAGTTATAGTTCAATA
ATAAAGTTTTTTTTGCTGATATTGTGGCTTCCCAG 3'
- PRIMER 2
5' CCCGGGTCTAGAAATGTACTTTCTTA
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Figure 4

00E230 04820960

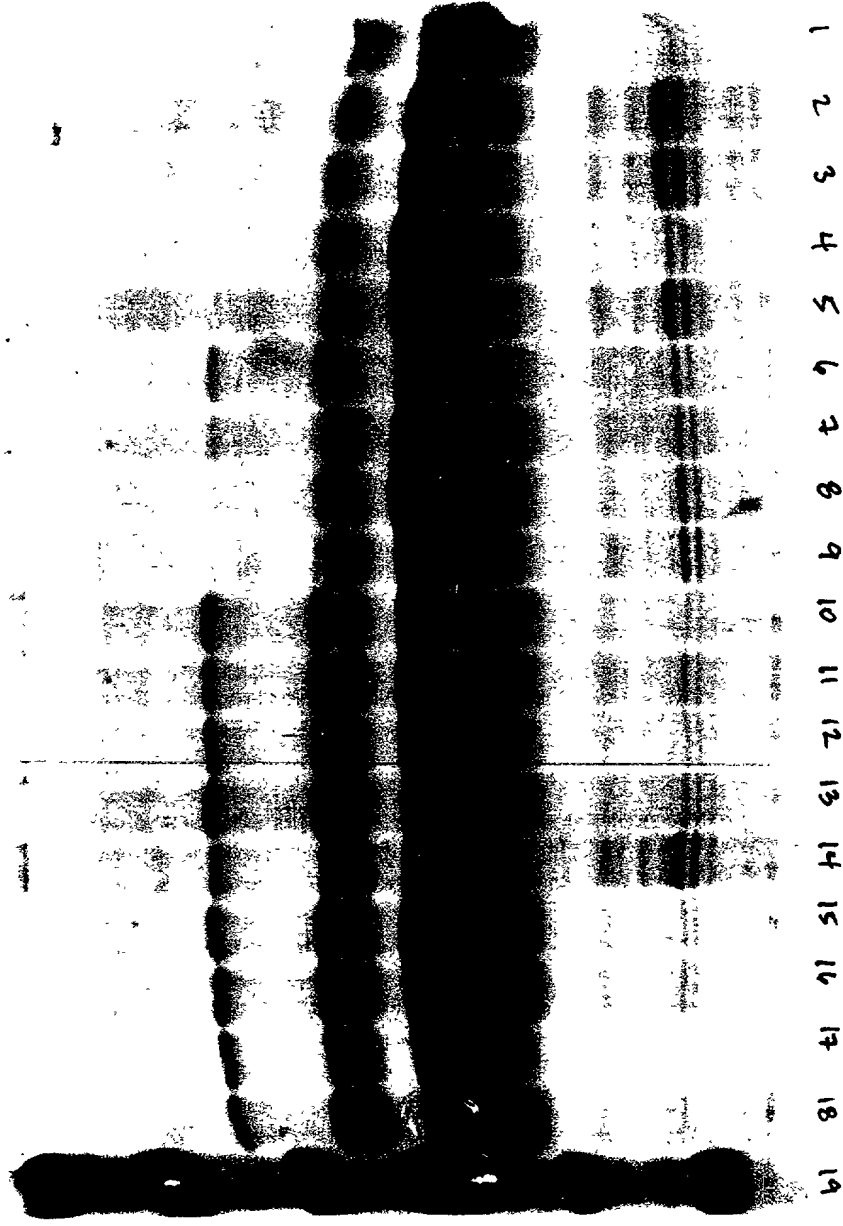


Figure 5

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

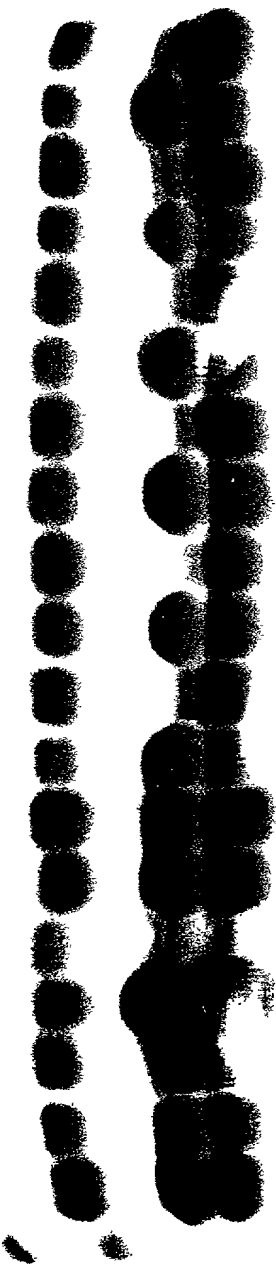


Figure 6

09502840.052300

A dark, heavily textured surface, possibly a book cover or a scan of a dark page. On the right edge, there is a vertical strip of numbers from 1 to 20, arranged from top to bottom. The numbers are white and stand out against the dark background. The overall image is very dark and grainy, with horizontal streaks and noise throughout.

7

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



Figure 8

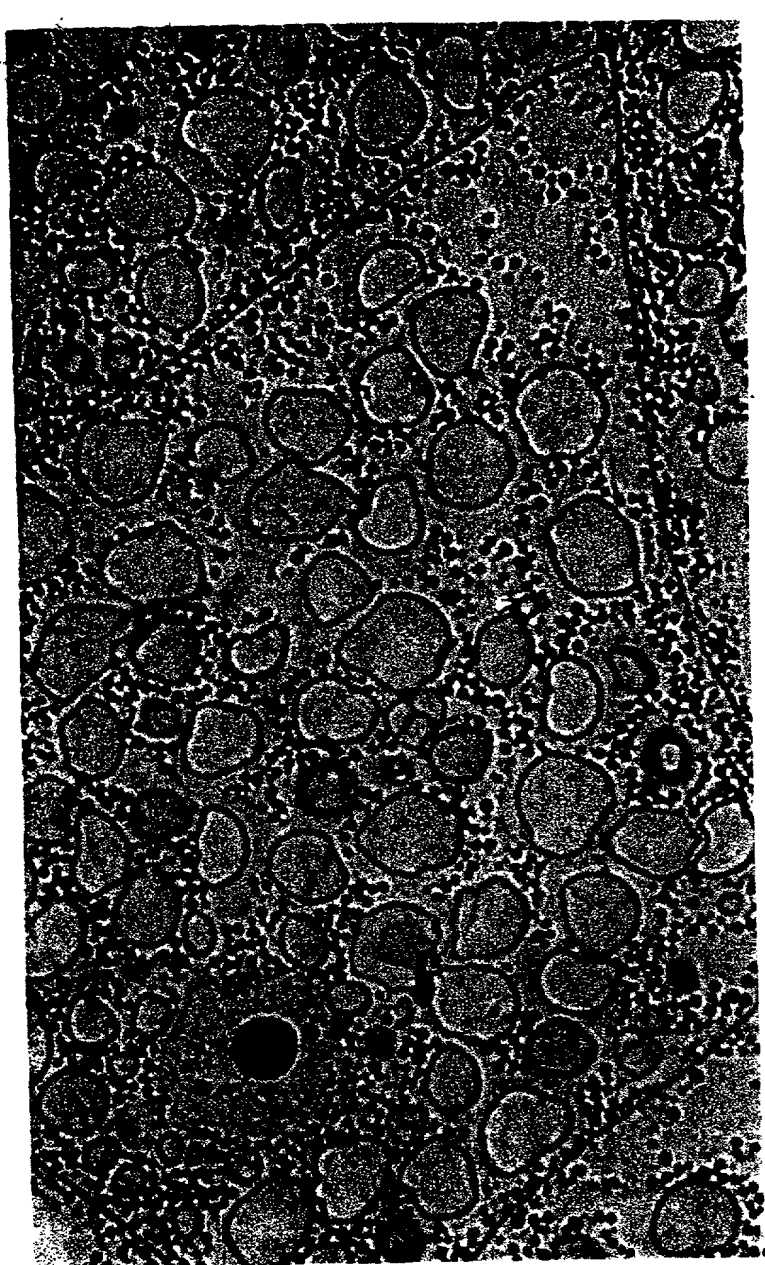


Figure 9

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

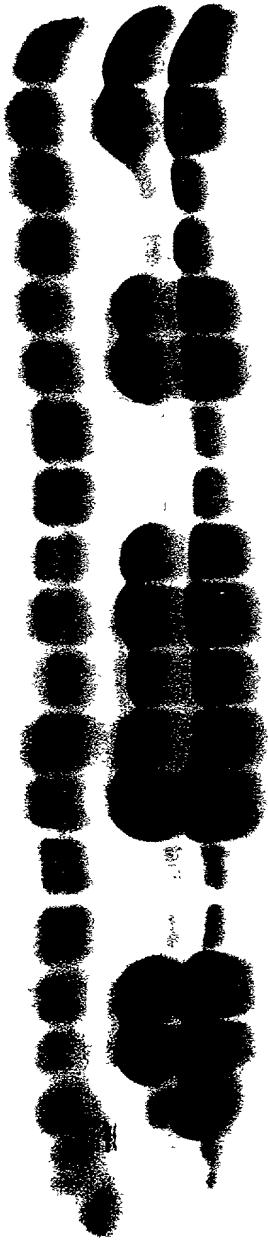


Figure 10

09502840, 062300

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: METHOD FOR ALTERING THE NUTRITIONAL CONTENT OF PLANT SEED.

The specification of which was filed on December 9, 1996, as application serial number 08/763,704.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (attached hereto).

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent of inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

No such applications have been filed.

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

No such applications have been filed.

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

No such applications have been filed.

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

Anglin, J. Michael	Reg. No. 24,916	Embretson, Janet E.	Reg. No. 39,665	Lempia, Bryan J.	Reg. No. 39,746
Bianchi, Timothy E.	Reg. No. 39,610	Fogg, David N.	Reg. No. 35,138	Litman, Mark A.	Reg. No. 26,390
Billig, Patrick G.	Reg. No. 38,080	Forrest, Bradley A.	Reg. No. 30,837	Lundberg, Steven W.	Reg. No. 30,568
Billion, Richard E.	Reg. No. 32,836	Holloway, Sheryl S.	Reg. No. 37,850	Schwegman, Micheal L.	Reg. No. 25,816
Brennan, Thomas F.	Reg. No. 35,075	Kalinowski, Leonard J.	Reg. No. 24,207	Slifer, Russell D.	Reg. No. 39,838
Burke, John E.	Reg. No. 35,836	Klima-Silberg, Catherine I.	Reg. No. 40,052	Viksins, Ann S.	Reg. No. 37,748
Clark, Barbara J.	Reg. No. 38,107	Kluth, Daniel J.	Reg. No. 32,146	Woessner, Warren D.	Reg. No. 30,440
Dryja, Michael A.	Reg. No. 39,662	Lemaire, Charles A.	Reg. No. 36,198		

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization/who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Schwegman, Lundberg, Woessner & Kluth, P.A. to the contrary.


Please direct all correspondence in this case to Schwegman, Lundberg, Woessner & Kluth, P.A. at the address indicated below:

P.O. Box 2938, Minneapolis, MN 55402
Telephone No. (612)339-0331

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature: 
Julie A. Kiriara

Date: 2/28/97

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Post Office Address: **1850 Roselawn Avenue**
Falcon Heights, MN 55113

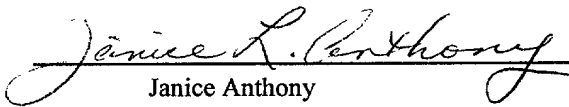
Residence: **Falcon Heights, MN**

Signature: 
Kenneth A. Hibberd

Date: 6/3/97

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Citizenship: **United States of America**
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Wakefield, RI 02879

Residence: **Wakefield, RI**

Signature: 
Janice Anthony

Date: 2/14/97

Full Name of inventor:
Citizenship:
Post Office Address:

Residence:

Signature: _____

Date: _____

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
- (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
 - (i) Opposing an argument of unpatentability relied on by the Office, or
 - (ii) Asserting an argument of patentability.

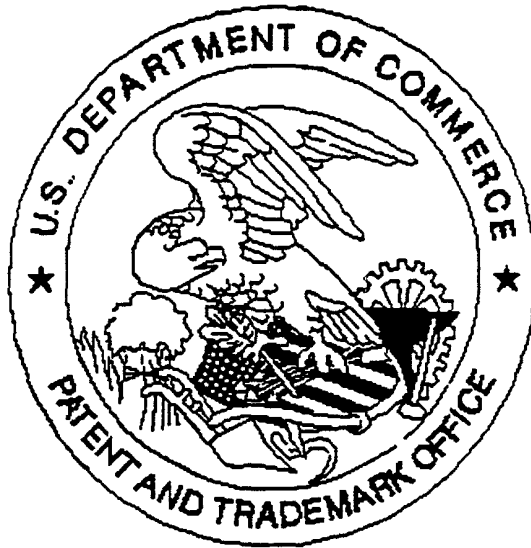
A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

(c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:

- (1) Each inventor named in the application;
- (2) Each attorney or agent who prepares or prosecutes the application; and
- (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

(d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.

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